STAPHYLOCOCCAL BACTERIOPHAGES

II. BACTERIOPHAGE ABSORPTION BY STAPHYLOCOCCI.

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In the process of bacteriophage multiplication in susceptible micro-organisms, the preliminary step involves the absorption of one or more phage particles on to the organism. Burnet (1934) has shown that, for the coli, dysentery and salmonella groups, the ability to absorb phage is intimately correlated with the structure of the surface polysaccharides of these organisms, and that resistance to phage action is correlated with inability to absorb phage. In a study of staphylococcal bacteriophages, Burnet and Lush (1935) found that the phages which they designated as "strong" and "intermediate" were all absorbed on normal strains of *Staphylococcus aureus*, irrespective of whether they lysed these strains or not. Rakieten and his co-workers (1936, 1937, 1938) in a further series of studies on the absorption of staphylococcal phages confirmed this non-specificity and also found that a number of unrelated organisms such as enterococci and certain members of the Bacillus group would absorb staphylococcal phages. Recently, in a paper published since the work reported here was done, Henry and Henry (1946) described the absorption of a polyvalent staphylococcal bacteriophage on to resistant variants of a strain of *Staphylococcus aureus*, these variants being produced as secondary growth after the parent culture had been lysed by phage.

The use of a series of phages for the typing of staphylococci, as developed by Wilson and Atkinson (1945), has led to further interest in the staphylococcal phages. A study of their apparently complex relationships may throw some light on the mechanism of phage multiplication and its relationship to the chemical and antigenic structure of the staphylococci.

The observations reported in this paper are concerned chiefly with the absorption of staphylococcal phages by intact staphylococci and by chemical fractions obtained from these organisms.

MATERIALS AND METHODS.

Phage filtrates were prepared by growing the phage on the appropriate staphylococcus on the surface of 1·2 p.c. nutrient agar plates, containing 0·2 p.c. glucose. When confluent lysis had taken place, the agar was cut out with a sterile scalpel, broken up in sterile broth and left in the refrigerator overnight before filtration through a Seitz E.K. filter. Filtrates prepared in this way have a titre of 10^{10} to 10^{12} particles per ml.

Phage counts were made in duplicate by plating 0·02 ml. of the appropriate dilution (made in nutrient broth) on the surface of quadrants of agar plates, previously inoculated with the propagating strain of staphylococcus.

In general, absorption tests were carried out at 37° C., sufficient phage being used in the absorbing mixtures to give from 50 to 200 plaques when plated.

The phages and cultures used may be divided into three groups, as follows:

1. Those phages acting on coagulase-positive staphylococci of human origin and forming a serologically homogeneous group. All, except one, were inhibited by a concentration of 0·25 p.c. sodium citrate. No phage in this group lysed any coagulase-negative staphylococci tested. This group comprised the phages and cultures isolated by Wilson and Atkinson (1945), which are used for typing pathogenic staphylococci. The various phage types of staphylococci

differ in their susceptibility to lysis by this group of phages, some being lysed by only one phage in the group, others being lysed by a number of phages. The staphylococcal strains vary in their ability to produce α and β toxins and some are non-haemolytic. Their degree of pigment production is also variable, ranging from albus to deep aureus. They were obtained from Professor G. S. Wilson and Dr. V. D. Allison. In addition, four cultures and phages isolated in this laboratory were found to fall into this group. Most of the work was done with this group.

2. A phage, X2, of bovine origin, obtained from Dr. Allison. This phage was serologically distinct from those of the first group and has not been found to lyse any staphylococci of human origin. It has been tested against four strains of animal origin, obtained from Dr. E. V. Keogh, and was found to lyse none of them. It is inhibited by 0·25 p.c. sodium citrate.

3. A phage, Warner, isolated in this laboratory by Dr. A. Palmer from a urine infected with a coagulase-negative *Staphylococcus albus*. It is serologically distinct from the other two groups and is not inhibited by 1·5 p.c. sodium citrate. It was found to lyse 30 p.c. of strains of coagulase-negative staphylococci tested.

EXPERIMENTAL RESULTS.

Absorption of Phage by Heat-killed Cultures.

The first series of absorption tests was made with broth cultures killed by boiling for five minutes. These were mixed with various phages selected from the first group and with the phages X2 and Warner. The absorptions were carried out at 37° C. for one hour and then the mixtures were plated on the appropriate propagating strain of staphylococcus. A control tube of phage and broth was included in each series and the percentage reduction in the plaque count calculated. Table 1 sets out the results obtained with a selected series of cultures and four different phages. It shows the origin of the staphylococci used and their coagulase and toxin producing state. Phage 33/35 lyses cultures 3 and 4 only and phage 51/145 lyses culture 145 only. Phage 33/35 is citrate insensitive; phage 51/145 is citrate sensitive. Phage X2 lyses only culture X2 and phage Warner lyses only culture S46. The results are expressed as percentages of phage inactivated rather than as the actual plaque counts obtained since the tests were done over a period of time and the counts on the control plates varied from time to time, although the concentration of phage used in each absorbing mixture was usually between 2,500 and 10,000 particles per ml.

The Table shows that there is no relationship between ability to inactivate phage and the presence or absence of α or β toxin. The phages lysing coagulase-positive human staphylococci are completely absorbed by all coagulase-positive staphylococci tested, irrespective of whether they lyse the organisms or not. Phage 51/145 has been tested against all the staphylococcal phage type cultures with similar results. In addition, all other phages of this group which have been examined show the same phenomenon. There are no cross reactions between the coagulase-negative and coagulase-positive phages and staphylococci. The results obtained with the bovine phage X2 and the coagulase-positive strains indicate that there are differences between the animal and human strains which are reflected in the partial inactivation of this phage with certain human cultures. The absorption tests, therefore, support the division of the cultures into three groups on the grounds of their origin, coagulase production, lysis by phage and the serological differences in the phages lysing them.

TABLE 1.

Percentage inactivation of staphylococcal bacteriophage by heat-killed cultures of staphylococci after 1 hour at 37° C.

Staphylococcus culture No.	Origin.	Coagulase.	Toxin.	P.c. inactivation of phage. 33/35.	51/145.	X2.	Warner.
3	Human	+	β	100	100	66	0
4	Human	+	α	100	100	69	0
18	Human	+	α	100	100	84	0
33	Human	+	α	100	100	60	0
145	Human	+	α	100	100	100	0
211	Human	+	αβ	100	100	75	0
373	Human	+	α	100	100	65	0
987	Human	+	α	100	100	91	0
1312	Human	+	Nil	100	100	53	0
1338	Human	+	Nil	100	100	—	0
X2	Bovine	+	αβ	95	100	100	0
G12	Bovine	+	αβ	100	100	100	0
603	Canine	+	β	100	100	—	0
S46	Human	—	Nil	0	0	0	100

Speed of Phage Absorption.

Apart from the information obtained on the relationships of the phages and staphylococci used in this series of absorption tests, the results reported above also showed that the union between phage and the killed staphylococcal cell is irreversible. Once the phage particle has been absorbed on a dead cell it is no longer free to infect when brought into contact with living cells susceptible to lysis. Mixtures of heat-killed cultures and phage may therefore be examined for the speed of the phage absorption by plating samples at suitable intervals. The speed of the absorption depends on the concentrations of the two reactants and on the temperature at which the absorption is carried out. No extensive study of the dynamics of the reaction has been made since the purpose of the experiments was to establish a suitable time period of incubation for the absorption tests.

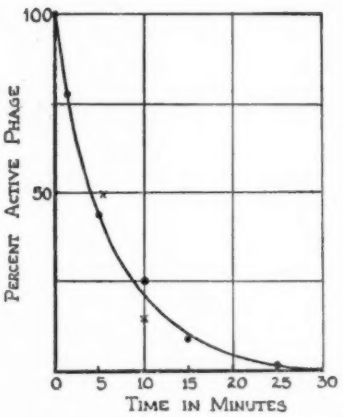

Fig. 1. Inactivation of phage 51/145 by a heat-killed culture of Staphylococcus 145 at 37° C.

The speed of the absorption at 37° C. was examined using phage 51/145 and culture 145. Equal volumes of suitably diluted phage were mixed with equal volumes of a heat-killed broth culture diluted 1 in 10 with broth. Samples were removed and plated at intervals. With a phage content of 1,750 particles per ml. absorption was complete in 20 minutes; with a concentration of 5,300 particles per ml. no phage could be recovered at the end of 30 minutes. Figure 1 shows the curve obtained when the percentage of active phage is plotted against time.

A standard period of one hour at 37° C. was therefore adopted as adequate for the absorption of the phages when using either cultures or chemical fractions obtained from them.

Absorption of Phage by Living Cultures.

If a phage lysing a coagulase-positive human strain is added to a young actively-growing broth culture which is insusceptible to lysis by the phage used but which absorbs it when heat-killed, the phage is quickly inactivated. In a typical experiment, phage 51/145, suitably diluted was added to an equal volume of a 5-hour glucose broth culture of 211. The mixture was incubated at 37° C. and at intervals samples were plated on to culture 145. If a light suspension of the resistant culture is used, the presence of some resistant cells does not interfere with the plaque formation by the unabsorbed phage particles when they are plated on the susceptible strain. Table 2 shows the plaque counts and percentage inactivation of phage at times up to 30 minutes. The plaque count made before mixing the phage and culture is twice the value expected on mixing, owing to dilution of the phage with the added culture. This is taken into account in the calculation of the percentage reduction of the phage.

It is apparent from the counts obtained that the living resistant culture absorbs the phage in the same way as do heat-killed susceptible or resistant organisms of the same group.

TABLE 2.

Reduction in infectivity of phage 51/145 in contact with a living culture of 211.

Time after addition of culture.	Mean plaque count.	P.c. inactivation.
Control (no culture)	107	
1 minute	35·5	34
5 minutes	22·5	57
10 minutes	13·5	75
15 minutes	5·5	90
20 minutes	6	90
25 minutes	1	98
30 minutes	0	100

Effect of Sodium Citrate on Phage Absorption.

Andrewes and Elford (1932) showed that, when a coli phage is added in excess to a culture of *B. coli* in the presence of sodium citrate, the bacteria are killed by absorption of the phage, although the phage itself does not multiply in the presence of citrate. Since certain of the

staphylococcal phages used in this investigation are inhibited on agar media in the presence of 0·25 p.c. sodium citrate, it was thought of interest to determine whether actual absorption of these phages on the staphylococcal cell took place in the presence of sodium citrate. The citrate-sensitive phage 51/145 and its specific culture 145 were used. Phage was added to heat-killed and living broth cultures of 145 in the presence of 0·5 p.c. sodium citrate. The experiment was arranged so that the final concentration of phage was the same in all tubes. Controls included phage plus broth, phage plus citrate, and phage and living culture without citrate. The mixtures were incubated at 37° C. and samples plated in duplicate after 60 and 90 minutes. The counts obtained are shown in Table 3.

TABLE 3.

Phage counts of 51/145 obtained after absorption on to cells in presence of 0·5 p.c. sodium citrate.

Tube No.	Contents of absorption tubes in ml. Phage.	Broth.	2·6 p.c. Na citrate.	Living culture.	Killed culture.	Plaque count. 60 mins.	90 mins.
1	0·8	1·2	—	—	—	54, 62	71, 50
2	0·8	0·8	0·4	—	—	52, 51	44, 45
3	0·8	—	0·4	0·8	—	12, 18	12, 11
4	0·8	—	0·4	—	0·8	5, 3	10, 9
5	0·8	0·4	—	0·8	—	64, 44	400+, 400+

It will be seen that, at the end of 90 minutes, the phage in the control tube No. 5 containing living organisms, and no citrate, had multiplied in the normal manner, giving an uncountable number of plaques on plating. With the living culture in the presence of citrate, the multiplication of the phage was inhibited and not only had it been inhibited but the actual number of infective phage particles had been reduced by 80 p.c. The same reduction also occurred with the heat-killed organisms. There is a suggestion that the phage in tube No. 2 containing citrate may have had its count reduced slightly at the end of 90 minutes, but this count is just within the limits of experimental error and, from other experiments, it may be stated that sodium citrate has no direct effect on the staphylococcal phage. The citrate and killed-culture tube did not show 100 p.c. reduction in phage activity as might have been expected from previous absorption tests with killed cultures, but this was probably due to the use of a rather light suspension of organisms. However, the results show that the effect of the citrate is not to inhibit phage absorption on the living or dead bacterial cell but that it prevents multiplication of the absorbed phage. It is apparent that, within the time limits of the experiment, this effect is an irreversible one, since the phage-infected living cells, when transferred for counting purposes to agar plates with consequent dilution of the citrate, still do not lyse and produce visible plaques.

Variation in Staphylococcal Cultures and its Effect on their Susceptibility to Bacteriophage Lysis.

The staphylococcal cultures in use from day to day in this work were grown in 0·15 p.c. glucose broth and it was noted that many of these broth cultures, if left on the bench for a few days, showed a tendency to become granular. Certain strains have shown this appearance regularly over a period of twelve months, while with others it has been of more infrequent occurrence. Both coagulase-positive and coagulase-negative strains have shown this change.

When broth cultures showing this granularity, which is associated with the development of rings at the surface of the broth and with decreased turbidity, are plated on blood agar, two types of colony may appear. One is the normal smooth butyrous colony of the staphylococcus. This type produces varying amounts of pigment and of α and β haemolysin, depending on the characteristics of the given strain. The variant type is smaller than normal, smooth, usually non-pigmented and extremely tenacious and sticky. When touched with a loop, it will usually come away from the plate in its entirety and it is difficult to emulsify. Such colonies, when inoculated into glucose broth, produce granular growth and surface rings and there is no tendency for them to revert to the normal smooth type on subculture. When these variants are derived from parent strains producing α haemolysin, there is a tendency for such strains to lose their haemolytic activity but this loss is not invariable. When derived from coagulase-positive parent strains, the variants retain their plasma-coagulating ability. Viscid variants similar to these were described by Bigger, Boland and O'Meara (1927) and it would appear that this variation is analagous to the smooth → rough variation so well known with other bacterial species.

The behaviour of these viscid variants towards phages lysing the parent strain is of particular interest. A number of these variants produced by the coagulase-positive human strains were tested for their phage susceptibility and it was found that they were completely resistant to lysis by phages lysing the parent strains. However, in spite of their resistance to lysis, these variants still retained the power to absorb the phages.

In Table 4 are shown the results of absorption tests with resistant viscid variants from three different strains. These were tested against their homologous phages and also against a phage which did not lyse but which was absorbed by the parent strains. A heat-killed normal smooth strain was included in each test as a control.

TABLE 4.

Absorption of phage by smooth cultures of staphylococci and by viscid variants.

Phage.	Culture.	State of culture.	Lysis.	Phage count after absorption.
3A/284	284	Smooth	+	2, 0
	284	Viscid	—	2, 1
	Nil			+++, +++*
47A/761	761	Smooth	+	38, 48
	761	Viscid	—	47, 42
	Nil			+++, +++
42E/1670	1670	Smooth	+	87, 81
	1670	Viscid	—	103, 110
	Nil			+++, +++
14/17	284	Viscid	—	2, 1
	761	Viscid	—	15, 13
	1670	Viscid	—	10, 5
	Nil			385, 376

* +++ = semi-confluent lysis.

The results showed that the viscid variants absorbed both homologous and heterologous phages to the same extent as did the parent strains. It is concluded that the smooth → viscid variation involves the loss from the staphylococcal cell of a component which is essential for the multiplication of the phage but which is not concerned with the preliminary step of absorption of the phage on the cell.

Inactivation of Phage by Watery Lysates of Staphylococci.

Rakieten *et al.* (1936) showed that cell-free autolysates of staphylococci possessed inactivating power for phages to which the living cultures were susceptible. They were unable to demonstrate phage inactivation by extracts from resistant staphylococci. One resistant culture inactivated all phages added to it but extracts of this culture were devoid of activity.

Lysates have been prepared from a number of the coagulase-positive human strains and their phage inactivating power examined. The results showed that lysates from strains of coagulase-positive human staphylococci inactivated all the phages of this group, that there was no inactivation by these lysates of the phage lysing the coagulase-negative human staphylococcus, and that such lysates possessed little neutralizing power for the phage of animal origin.

Lysates were prepared by the method of Burnet (1934) except for the fact that filtration was omitted. The organisms were grown on nutrient agar in Roux bottles for 48 hours at 37° C. The growth on each bottle was washed off with 5–10 ml. of sterile distilled water and the resulting suspensions left in the water bath at 56° C. for two days while autolysis took place. The lysate was then spun at 3,000 r.p.m., to remove the bulk of the organisms. Final centrifugation in a high speed centrifuge at 12,000 r.p.m. for 20 minutes resulted in the separation of a clear straw-coloured supernatant which was then tested for its phage inactivating activity.

TABLE 5.

Inactivation of phage 51/145 by lysates of staphylococci.

Phage.	Lysate.	Dilution of lysate.	Plaque count.	P.c. inactivation.
51	145	1 in 2	2, 1	98
51	145	1 in 20	10, 15	88
51	145	1 in 200	88, 84	10
51	Nil		99, 95	—
51	373	1 in 2	3, 1	98
51	373	1 in 20	36, 51	55
51	373	1 in 200	90, 79	10
51	Nil		93, 97	—

In setting up the tests for inactivation of phage, the lysate was diluted in broth 1 in 10 and 1 in 100 and to these dilutions, together with another tube containing undiluted lysate, were added equal volumes of diluted phage. Plaque counts were made after incubation for one hour at 37° C.

In one experiment, lysates were prepared from cultures 145 and 373, and tested against phage 51/145; 145 is lysed by this phage, while 373 is not. The results of the plaque counts obtained, together with the percentage inactivation of the phage, are shown in Table 5.

The lysate from culture 373 was not as active as the lysate from 145. Nevertheless, it was apparent that the lysate did possess the power of inactivating the phage in the same way as do the heat-killed or living cultures of this strain.

Lysates have been prepared also from cultures 3 and 211 and tested against phage 51/145 and have been found to have orders of activity comparable with lysates of 145 prepared at the same time. Lysates of 145 have been tested for phage-inactivating power for phages X2 and Warner. No inactivation of phage Warner takes place in the presence of an equal volume of undiluted lysate of 145. In the case of phage X2, which showed only partial absorption of some of the heat-killed cultures of the human coagulase-positive strains, although 145 absorbed it completely, there was only partial inactivation of the phage by the lysate of 145. Table 6 shows the results of the absorption of phages 51/145 and X2 by a lysate of 145. This lysate, it will be noted, is of greater activity for phage 51/145 than the one used in Table 5.

TABLE 6.

Inactivation of phages 51/145 and X2 by a lysate of staphylococcus 145.

Phage.	Lysate.	Dilution of lysate.	Plaque count.	P.c. inactivation.
51	145	1 in 2	0, 0	100
51	145	1 in 20	0, 0	100
51	145	1 in 200	56, 56	50
	Nil		86, 134	—
X2	145	1 in 2	50, 84	46
X2	145	1 in 20	76, 110	33
X2	145	1 in 200	44, 100	49
	Nil		150, 130	—

Chemical Nature of the Phage Inactivating Agent of Staphylococcal Lysates.

Various methods of chemical fractionation of the lysates were carried out in an attempt to separate the component responsible for phage inactivation. Rakieten and Tiffany (1938) found that protein fractions from whole cultures of staphylococci possessed phage-inactivating activity. On the other hand, Burnet and Lush (1935) stated that staphylococcal phage is specifically bound to an antigenic complex from which the polysaccharide haptene can be derived. Working with one of their phages, Freeman (1937) found that precipitation of nucleoprotein from extracts prepared by grinding staphylococci in a ball mill removed the phage-inactivating activity of the extract. By tryptic digestion of this nucleoprotein complex, polysaccharide material was obtained which possessed phage-inactivating power.

It was found that polysaccharide extracted from whole broth cultures by Fuller's method (1939) for the preparation of streptococcal group-specific polysaccharide was devoid of activity. As this method is a drastic one, milder methods were tried.

TABLE 7.

Inactivation of phage 51/145 by the alcohol-precipitated fraction of lysates of staphylococci 145 and 373.

Phage.	Fraction.	Dilution of fraction.	Plaque count.	P.c. inactivation.
51	145	1 in 2	7, 7	90
	145	1 in 20	12, 14	83
	145	1 in 200	44, 40	45
	373	1 in 2	4, 3	95
	373	1 in 20	4, 8	90
	373	1 in 200	40, 38	49
	Nil		74, 78	—

Experiments showed that the active fraction was precipitated from the lysates by the addition of five volumes of absolute alcohol plus a few drops of glacial acetic acid. If such preparations were left in the refrigerator overnight, a fine white flocculer precipitate settled out which could be readily removed by centrifugation. On resuspending the precipitate in distilled water, portion of it went into solution. Adjustment of the pH to slight alkalinity, using N/10 NaOH, resulted in solution of the major portion of the precipitate, and the insoluble residue was removed by centrifugation. When the supernatant was tested for phage-inactivating activity, it was found that the activity was of the same order as that of the original lysate. In Table 7 are shown the results obtained when such fractions from lysates of 145 and 373 were tested against phage 51/145. The original phage-inactivating activity of these lysates was given in Table 5.

In this experiment the precipitates from 15 ml. of lysate were redissolved in 3 ml. of distilled water. The results show that there was little loss of activity by alcohol precipitation; the increased titre of from 10 p.c. to 45 p.c. with the 1 in 200 dilution presumably being due to the concentration of the original volume.

In an attempt to separate the polysaccharide and protein fractions contained in the material precipitated by five volumes of alcohol, a number of lysates was prepared and subjected to various manipulations. It was found that the major portion of the active fraction could be reprecipitated from the solution of the alcohol-precipitated material by the addition of 2·5 volumes of acid alcohol. This precipitate gave a turbid emulsion on the addition of distilled water and could be brought into solution by adjusting the pH to slight alkalinity. It gave a positive Biuret reaction and a negative Molisch reaction, and was apparently protein in nature. The material not precipitated by 2·5 volumes of alcohol was precipitated by the addition of six volumes of acetone. This acetone precipitate was completely soluble in distilled water. It gave a negative or faint positive biuret and positive Molisch reaction and appeared to be polysaccharide. Fractions were prepared by this method from cultures 3, 145 and 211 and tested against phage 51/145.

TABLE 8.

Inactivation of phage 51/145 by fractions of lysate of staphylococcus 3.

Phage.	Fraction of culture 3.	Dilution of fraction.	Plaque count.	P.c. inactivation.
51	Original lysate	1 in 2	0, 0	100
		1 in 20	0, 0	100
		1 in 200	0, 0	100
	2·5 volume alcohol ppt.	1 in 2	0, 0	100
		1 in 20	0, 0	100
		1 in 200	0, 0	100
	Acetone precipitate	1 in 2	50, 51	9
		1 in 20	52, 54	5
		1 in 200	69, 43	0
	Nil		56*	

* Mean of 10 counts.

Table 8 shows the results obtained when these fractions and the original lysate from culture 3 were tested. Similar results were obtained with the fractions from 145 and 211. Fractions precipitated by acetone were almost completely devoid of activity, while the fractions which apparently consisted of protein were as active as the original lysates.

It was possible that the lack of activity in the acetone precipitate was due to the action of the acetone and not to the fact that the active material had already been removed by the precipitation with 2·5 volumes of alcohol.

Another batch of lysate 145 was precipitated with 5 volumes of alcohol, the precipitate redissolved and the supernatant after reprecipitation with 2·5 volumes of alcohol was treated with sufficient alcohol to give a final concentration of five volumes. This treatment resulted in the precipitation of floccular material which was removed by centrifugation and redissolved in distilled water. When this fraction and the precipitate with the 2·5 volumes of alcohol were tested for phage-inactivating activity, the former was found to be almost devoid of activity, while the "2·5 volume" fraction was active.

The conclusion was therefore reached that the phage-inactivating agent was either protein in nature or closely associated with the protein. No differences could be detected in the ability of fractions from cultures 3, 145 or 211 to neutralize phage 51/145, indicating that this component is common to all the coagulase-positive human staphylococci. Presumably it is also the component on which the phages are absorbed in the intact cell. Further evidence in favour of the protein

nature of the phage-inactivating agent was found by extracting crude lysates with chloroform. If 0·25 volumes of chloroform and a few drops of caprylic alcohol were added to a lysate and the mixture shaken in a mechanical shaking machine for half-an-hour and then centrifuged, the protein gel so formed was found to contain the active agent. This could be recovered from the gel by precipitation with alcohol. Several such extractions were found to be necessary to render the lysate devoid of inactivating power.

It was thought possible that the active agent might be associated with a nucleoprotein. Sevag, Lackman and Smolens (1938) working with streptococcal nucleoproteins showed that the bond between the nucleic acid and the protein in fractions prepared from these organisms could be broken by treatment with sodium carbonate at 50–55° C. and that the protein could then be removed from the solution by the chloroform gel method. This treatment was, therefore, applied to the protein fractions isolated from cultures 3, 145 and 211. An equal volume of 10 p.c. aqueous sodium carbonate solution was added to a solution of the fraction precipitated with 2·5 volumes of alcohol, and the mixture was left in the water bath at 56° C. for an hour. The mixture was then shaken with 0·25 vols. of chloroform and a few drops of caprylic alcohol for an hour. After centrifugation, the chloroform gel and the supernatant were separated and each precipitated with 2·5 volumes of alcohol. After solution in distilled water, the precipitates were tested for phage-inactivating activity against phage 51/145. In all cases, the supernatant contained the major portion of the active agent. There was also slight activity in the chloroform gel fraction, but as it was found impossible to separate all the aqueous supernatant from the gel, it was considered that this activity was probably due to small amounts of the supernatant entrained in the gel.

It was concluded that the phage-inactivating agent was associated in the lysates with a nucleoprotein, and that, on breaking the bond between the nucleic acid and the protein, the nucleic acid still retained the power to inactivate phage.

The nature of this nucleic acid is a matter of interest. McCarty and Avery (1946) have described an improved method for the isolation of the desoxyribonucleic acid of the pneumococcus, which makes use of the fact that this substance is precipitated by 1 p.c. calcium chloride in the presence of 0·2 volumes of alcohol. This method of precipitating desoxyribonucleic acid was applied to the fraction precipitated by 2·5 volumes of alcohol from a lysate of culture 211. To the fraction dissolved in distilled water were added 0·2 volumes of absolute alcohol and 0·1 volumes of a 10 p.c. aqueous calcium chloride solution. On standing, a fine granular precipitate was thrown down. This was removed by centrifugation and redissolved in distilled water. The supernatant and this fraction were reprecipitated with alcohol, resuspended in distilled water, and tested for phage-inactivating activity with phage 51/145. The fraction not precipitated with calcium chloride retained the phage-inactivating power of the original material, suggesting that any desoxyribonucleic acid present in the lysate in the free state is not involved in the phage inactivation.

The results suggest that it is the ribonucleic acid of the staphylococcal cell, occurring in close association with protein in the lysates and presumably in the intact cell, which is the phage-inactivating agent. The fact that heat-killed cells in which the protein has presumably been denatured absorb phage as readily as do living cells, lends support to this conclusion.

The work of Henry and Stacey (1943) and of Bartholomew and Umbreit (1944) on the significance of magnesium ribonucleate in the staining of Gram-positive bacteria suggested that removal of the Gram positiveness of the staphylococcus by the action of bile salts might also remove the ability of the organism to absorb phage. Several of the coagulase-positive strains were treated according to their methods, using solutions of sodium desoxycholate, sodium taurocholate or sodium cholate, but all attempts to convert the organisms to the Gram-negative state were unsuccessful. No explanation of this failure can be offered.

DISCUSSION.

The results of the phage absorption tests with intact cells, with lysates and with fractions of the lysates, point to the significance of nucleoprotein in the absorption of phage by the staphylococcal cell. The inter-relationships between the staphylococci of various origins, as revealed by the absorption tests, suggest that coagulase-positive staphylococci of human origin, whether phage susceptible or not, all possess a common nucleoprotein component situated at the surface of the cells, and it is on to this that phage absorption takes place. It has not been found possible with the coagulase-positive strains studied to develop true phage resistant cultures, in the sense that such cultures have lost their ability to absorb phage. Burnet and Lush (1935) reported that they were able to do so only with difficulty. The results of Henry and Henry (1946), and the results reported in this paper on the absorption of phage by variant strains not susceptible to lysis, may therefore be attributed to the fact that the nucleoprotein component is the

expression of a very fundamental characteristic of the coagulase-positive staphylococci.

No work has been done on the nature of the phage-inactivating agent of the coagulase-negative strains, but, since there is no cross absorption with these strains and the coagulase-positive phages, it would appear that the specific nucleoprotein of the coagulase-positive staphylococci is absent from the coagulase-negative strains. Furthermore, it has been found that the culture Warner readily developed true phage-resistant variants.

There is increasing evidence of the occurrence of nucleoproteins of different biological specificities and it may be that slight differences in the nucleoproteins of the animal and human coagulase-positive staphylococci would explain the irregular or partial absorptions obtained with the bovine phage and the human strains.

Absorption of the phage particle on the staphylococcal cell having taken place, the phage is normally released from the cell only as a result of its multiplication therein and the consequent lysis of the cell. Under certain conditions, however, in the case of lysogenic strains, the union between the resistant cell and the phage can be reversed, since susceptible cells brought into contact with a lysogenic strain become infected with the phage and are lysed. Occasionally also the phage-cell equilibrium in a lysogenic strain may break down and the cells may be lysed.

The exact nature of the factors in the staphylococcal cell concerned in phage multiplication can, in the present state of our knowledge of the complex physiological processes of the bacterial cell, be only a matter for speculation. There are, however, a number of points which may serve to throw some light on the problem. One is the fact that, with certain phages, the presence of citrate inhibits multiplication. This effect can also be obtained in the presence of oxalate and it has been suggested that it is due to the removal of available calcium ions from the medium. It is known that the presence of calcium and magnesium is essential for the functioning of some bacterial enzyme systems, for example, the proteases of proteolytic organisms and the desoxyribonuclease of the pneumococcus. The process of multiplication of the phage may involve certain enzyme systems of the cell requiring calcium or magnesium. The fact that some of the staphylococcal phages are unaffected by the presence of citrate also suggests that alternative mechanisms of phage reproduction are available in the staphylococcal cell. It is also interesting to note the results of Cohen and Anderson (1946) on the action of 5-methyltryptophane as an inhibitor of the multiplication of T2 phage on *B. coli*, with its implication as to the importance of the indole nucleus in many phenomena of multiplication.

Burnet and Lush (1935) and Freeman (1937) found evidence that polysaccharide was concerned in the phage inactivation by the strains of staphylococci they studied. It has not been found possible to demonstrate any activity in the preparations of polysaccharide studied in the present work. It is possible that the methods of preparation employed did not permit the isolation of polysaccharide in an active state. The changes involved in the occurrence of viscid variants are analagous to the S → R change in other organisms and these changes are also correlated with loss of the ability to support the growth of phage. Duran-Reynals (1933) also showed that viscid variants were lacking in the spreading factor possessed by parent strains. Considerable difficulty is experienced in the serological typing of staphylococci by agglutinating sera if the strains tested have been isolated for some time, such strains reacting to a wide range of sera (Dr. Keogh, personal communication). This appears to be associated with the loss of more specific antigens which may be polysaccharide in nature. Correlation of the results of serological and phage typing and further examination of chemical fractions of the staphylococcus are clearly indicated.

SUMMARY.

Phage absorption tests, using heat-killed cultures of staphylococci, were made with 3 groups of phages:

(a) Those lysing coagulase-positive staphylococci of human origin;
(b) Those lysing coagulase-positive staphylococci of animal origin;
(c) Those lysing coagulase-negative staphylococci of human origin.

All phages lysing coagulase-positive staphylococci of human origin were absorbed by all coagulase-positive staphylococci, whether they lysed the organism or not.

Partial absorption of a bovine phage occurred with human coagulase-positive strains.

There was no reaction between coagulase-positive and coagulase-negative phages and cultures.

Absorption is complete in 30 minutes with a concentration of 5,000 phage particles per ml.

The presence of 0·5 p.c. sodium citrate inhibited multiplication of phage in living susceptible cultures but did not inhibit absorption.

Viscid variants of staphylococci were resistant to lysis by phages lysing the parent strains, but still retained their power to absorb phage.

Chemical fractions were prepared from lysates of the human coagulase-positive staphylococci and tested for phage-inactivating activity.

The results indicated that nucleoprotein, probably ribonucleic in nature, was concerned in the phage inactivation.

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THE "T" ANTIGEN OF GUINEA-PIG AND HUMAN RED CELLS

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The fact that samples of human blood cells contaminated with certain bacteria may be non-specifically agglutinated by human sera of any type has been well-know to haematologists for many years. The phenomenon was described by Thomsen (1926) and shown to be due to a bacterial enzyme by Friedenrich (1928). The serum component responsible, generally known as "T" agglutinin, is present to varying titre in practically all human and many mammalian sera. It can be readily removed by absorption with treated cells. Work in this laboratory, Burnet, McCrea and Stone (1946), Stone (1947) has shown that similar changes in cells are produced by viruses of the mumps-influenza group and that very active bacterial enzyme preparations can be obtained from cultures of *Vibrio cholerae*.

In this paper experiments are reported bearing on the nature of the change induced in the red cell by what we have termed the receptor-destroying enzyme (RDE) of the cholera vibrio. It will be shown that the altered cells develop on their surface an antigenically new component and that in the course of elution of the enzyme soluble antigenic material derived from the cell surface is liberated. The antigen from guinea-pig cells provokes the formation of agglutinins for normal cells and "T" agglutinins for altered cells. When the soluble material from human cells is used as antigen in rabbits only traces of normal agglutinins are produced but there is a free production of "T" agglutinins. There is some minor serological relationship between human and guinea-pig "T" agglutinins but in the rabbit at least they are clearly differentiable.

MATERIALS AND METHODS.

Human group O and guinea pig red cells were obtained from defibrinated blood and washed with normal saline.

High titre preparations of the receptor-destroying enzyme (RDE) were obtained from soft (1 p.c.) agar 16-hour cultures of *Vibrio cholerae*. The strain 4Z was chiefly used but other strains provided equally active material. The fluid was pressed out of the agar held in a gauze bag and clarified by centrifugation and passage through a Seitz EK filter.

Preparation of RD-cells and eluates. The standard procedure was to mix in the cold 0·5 ml. of packed red cells, 2·5 ml. of saline buffered at pH 7·0 with borate and containing 0·1 p.c. $CaCl_2$ and 4 ml. of active agar filtrate. As a rule 6 sets of mixtures were made in centrifuge tubes. These were held in ice water for a few minutes, rapidly centrifuged and after discarding the supernatant fluid the cells were washed once with 5 ml. of ice-cold buffered saline. The deposit was re-emulsified in 1 ml. of buffered saline and the tubes placed in a water bath for 1 hour at 37° C. to allow elution to take place into one quarter of the original volume.

After a final centrifugation the supernatant fluid, which should be almost or completely free of haemoglobin, was collected as eluate and the cells were washed once with saline and used as required. To determine whether the process of receptor destruction was complete the cells

[1]This work has been carried out under a grant from the National Health and Medical Research Council.

were tested with 10 agglutinating doses of the virus MIL B (influenza B) which comes at the end of the receptor gradient. All emulsions of cells used as RD cells in this work were completely unaffected by this virus.

Rabbits weighing 1·5 to 2 kg. were used for serum production. Two intravenous injections of 0·25 ml. packed cells or 1·0 ml. of eluates prepared as described, were given at a week's interval and the animals bled 10–14 days later. Sera were inactivated at 56° C. for 30 minutes and stored in the cold.

Agglutinin titrations were made in tubes of 1 cm. internal diameter. Doubling dilutions of 0·25 ml. volume were made in saline and an equal volume of 1 p.c. cell suspension in saline added. Readings were made after the cells had settled at room temperature, "one plus" partial agglutination as judged by the pattern of deposited cells being taken as the end point.

Absorptions were made using 0·5 ml. of packed washed red cells of appropriate type to 2·5 ml. of 1:100 serum. The mixtures were spun after 30 minutes at room temperature.

EXPERIMENTAL RESULTS.

None of the rabbit sera we have tested before immunization contained agglutinins for normal guinea-pig or human cells when tested at dilutions of 1:10 or higher. All, however, contained relatively low titre agglutinins ("T" agglutinins) acting on human or guinea-pig cells treated by RDE. The titres have ranged from 1:80 to 1:250 and are usually about equal for both species of cells. No tests were made with treated rabbit cells since these in our experience are spontaneously agglutinable.

TABLE 1.

Immunization of rabbits with normal and RD-guinea-pig cells.

		Titres against.			
		N.G. cells.		RD-G cells.	
Rabbit.	Immunized with.	I.	II.	I.	II.
A	N.G.	<10	2,400	600	2,400
B	N.G.	<10	4,800	120	4,800
C	RD-G	<10	1,600	250	4,800
D	RD-G	<10	600	200	3,200

ABSORPTION RESULTS.

		Titres against.	
Serum.	Absorbed with.	N.G.	RD-G.
B II	Unabsorbed	4,800	4,800
N.G.	N.G.	<100	400
	RD-G	150	100
D II	Unabsorbed	600	3,200
RD-G	N.G.	<100	2,400
	RD-G	<100	<100

Table 1 gives an abbreviated summary of the results of immunization with normal and RDE treated guinea-pig cells. Absorption results with sera A and C were almost identical with B and D respectively. These show that when RD-G cells are used for immunization the former "T" agglutinin titre against such cells is greatly raised and is not significantly reduced when the serum is absorbed with normal guinea-pig cells. Both normal and "T" agglutinins are removed by absorption with RD-G cells.

These results establish that the "T" agglutinogen is a definite immunological entity, that it is not present on the surface of normal guinea-pig cells and that it appears on the surface following treatment of the cells with RDE. Treated cells are still capable of provoking the formation of and of absorbing normal type agglutinin but it seems probable that a considerable proportion of the normal agglutinogen is replaced by the "T" agglutinogen.

Similar experiments with human cells have given essentially similar results, but the reduced ability of RD treated cells to produce agglutinin for normal cells is even more pronounced.

TABLE 2.

Immunization of rabbits with normal and RD-human cells.

Rabbit.	Immunized with.	Titres against. N.H. cells. I.	N.H. cells. II.	RD-H cells. I.	RD-H cells. II.
173	N-H	<10	3,200	40	20,000
174		<10	5,000	150	60,000
175		<10	5,500	150	30,000
170	RD-H	<10	350	40	22,000
171		<10	150	100	18,000
172		<10	800	300	20,000

ABSORPTION RESULTS.

Serum.	Absorbed with.	Titres against. N.H.	RD-H.
173 II	Unabsorbed	6,000	50,000
N.H.	N.H.	<100	1,600
	RD-H	180	400
174 II	Unabsorbed	6,000	60,000
N.H.	N.H.	120	3,600
	RD-H	3,000	600
172 II	Unabsorbed	1,500	20,000
RD-H	N.H.	<100	20,000
	RD-H	200	<100

Particular interest attaches to the experiments using eluates from human or guinea-pig cells. These sera were prepared primarily to obtain an anti-enzyme which might be of value in the analysis of RD action, the eluate being regarded as the purest available preparation of the enzyme. No anti-RDE action was demonstrable either in these or in any other of the sera we have prepared but with eluates from both human and guinea-pig cells active agglutinating sera have been produced. Four antisera against guinea-pig cell eluates and six against human cell eluates (4 group O, 2 group A) have been studied.

TABLE 3.

Agglutination tests with sera against eluates of RDE from human and guinea-pig red cells.

Serum.	Eluate from cells.	Titres against. N.G.	N.H.	RD-G.	RD-H.
E I	Guinea-pig	<10	<10	80	120
II		4,000	<10	15,000	250
J I	Human	<10	<10	125	120
II		<10	20	800	2,400
P I	Human	<10	<10	50	100
II		<10	300	100	8,000

Table 3 shows typical figures for three representative pairs of sera. The four sera against guinea-pig eluates all showed significant agglutination of normal guinea-pig cells, titres being 4,000, 4,000, 600, 300 while the titres for treated cells (RD-G) were 20,000, 15,000, 1,600 and 1,200 respectively. Sera against human cell eluates included one which showed a negligible rise with RD-H cells and no other change. The other five gave titres against normal human cells of 300, 120, 80, 20 and <10 with the corresponding RD-H titres 8,000, 4,000, 2,400, 2,400 and 1,600.

Absorption experiments gave results closely corresponding to those obtained with sera prepared by immunization with RD-G cells (Table 4).

There is apparently no very close relationship between the "T" agglutinogens of human and guinea-pig cells. Of the seven sera showing an active production of "T" agglutinins after immunization with eluates only one (J in Table 4) showed a significant rise in the titre of the heterologous "T" agglutinin. As a rule the other sera were slightly higher in titre than before immunization, but the rise did not exceed twofold.

TABLE 4.

Absorption of anti-eluate sera.

Serum.	Absorbed with.	Agglutinin titres against. N.H.	RD-H.	N.G.	RD-G.
F II	Unabsorbed	<10	400	4,000	20,000
G.P.	N.G.	...	600	<100	1,200
	RD-G	...	200	<100	<100
J II	Unabsorbed	20	2,400	<10	800
H	N.H.	<50	2,400	...	600
	RD-H	<50	<50	...	<50

When immunization was with RD treated human cells, there was a significant increase in RD-G agglutinins.

In the three rabbits shown in Table 2 as immunized with RD-H cells the changes in titre for RD-G cells were respectively 25–1,000, 50–1,200, 75–1,000. These titres are only about 1/20 of those against the homologous RD-H cells. Absorption with RD-H cells produce some reduction of RD-G titre but proportionately much less than the reduction in the homologous titre. Serum J (anti-eluate Table 4) was the only one in which complete or nearly complete absorption of "T" agglutinin for heterologous cells occurred.

Identity of Antigenic Changes Induced in Human Red Cells by RDE and by Viruses of the Mumps-Influenza Group.

In a recent paper, Stone (1947), it was shown that in all essential respects the action of RDE on human red cells followed the pattern set by the viruses of the mumps-influenza group. Both types of treated cell are agglutinated to appropriate titres by the "T" agglutinins of normal sera. It was of interest, therefore, to extend this comparison to the action of immune RD-H serum absorbed free of normal human cell agglutinins.

Washed group O human cells were treated at 37° C. with the following viruses: mumps, Newcastle disease, influenza (Melbourne M and Swine 15). Mumps and Newcastle disease cells were restabilized in two hours while Melbourne required six hours' exposure before the cells were stable. Swine influenza virus alone will never, in our experience, produce stable cells, so after two hours' exposure the cells were treated with an appropriate dilution of ferret immune serum to stabilize them and then washed twice with saline. In order to control the effect of serum, a similar treatment with the appropriate ferret immune serum was applied to cells treated with Melbourne virus. Both unstable (two hours' exposure) and stabilized (six hours' exposure) cells were used. The results in Table 5 show that virus-treated cells are agglutinated to significant titres by the RD-H agglutinin. The titre obtained is considerably reduced when treatment with immune serum is needed to prevent spontaneous agglutination and in no instance is as high as that found with RDE-treated cells. The lower levels presumably depend on the more limited exposure of new antigen produced by virus action.

TABLE 5.

Agglutination by RD-H antibody of virus treated cells.

Human cells treated with		Titre with sera R 172. Bleed I.	Bleed II (absorbed).
MU	2 hours	10	1,600
NDV	2 hours	50	5,000
Melbourne	6 hours	30	2,000
Melbourne	2 hours + I.S.	20	200
Melbourne	6 hours + I.S.	20	600
SW	2 hours + I.S.	60	1,000
RDE	1 hour	300	20,000

DISCUSSION.

It is clear from these results that under the action of the vibrio enzyme RDE a new antigenic component is exposed or developed on the surface of human or guinea-pig erythrocytes. The agglutinins developed when normal human cells are injected into rabbits correspond presumably to a considerable number of different antigens and no attempt has been made to analyse further this response.

The failure of normal cells to absorb RD-H or RD-G agglutinins, however, eliminates any of the normal surface constituents from being involved. There is in many of the sera a clear indication either that the available "normal" antigens have been reduced in amount in the treated cells or that the presence of the new antigen has interfered with the immunological response to other components still present.

The significance of the fact that eluates from treated cells are actively antigenic in the sense of producing agglutinins for normal or treated cells is not clear. Further investigation would be needed to determine whether this liberation of antigen is a necessary part of the reaction between the enzyme and the red-cell surface or whether it is only a secondary result of the changes initially produced. The failure of eluates from human cells to produce "normal" agglutinins perhaps suggests that the effect of the enzyme is not merely a simple uncovering of a pre-existent antigen.

The appearance of a new cellular antigen by the action of certain viruses or bacterial products on red cells raises the possibility that similar action *in vivo* might result in the production of auto-antibodies corresponding to the new antigen. An analogous possibility has been suggested as a likely explanation of blackwater fever in man. Fairly extensive experiments in guinea-pigs inoculated intracardially with viruses, vibrio enzyme and homologous treated cells have failed to produce more than trivial increases in "T" agglutinin. This would suggest that special conditions would be necessary in the human subject before such auto-immunization could develop as a result of infection by a micro-organism capable of producing red-cell damage of the type being considered.

SUMMARY.

Human or guinea-pig erythrocytes treated with the "receptor-destroying" enzyme of *V. cholerae*, when used for immunization of rabbits provoke the appearance of agglutinins in high titre which are not removed by absorption with corresponding normal cells,

These agglutinins also act on cells treated with viruses of the mumps-influenza group.

When the enzyme is allowed to elute from treated cells, the eluate contains in addition to the enzyme, antigenic material from the cells which is capable of inducing agglutinin of the new type.

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DESQUAMATION OF INTESTINAL EPITHELIUM *IN VITRO* BY *V. CHOLERAE* FILTRATES:

CHARACTERIZATION OF MUCINASE AND TISSUE DISINTEGRATING ENZYMES

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It has been shown by Burnet, McCrea and Stone (1946) that the action of viruses of the mumps-influenza group on red cells can be imitated almost completely by enzymes present in filtrates from cultures of *Cl. welchii* and *V. cholerae*. The "receptor-destroying" effect of the vibrio filtrates is a very potent one and it would obviously be of great interest to establish the chemical nature of the substrate involved. Numerous experiments have been made with this objective, so far without positive result. In the course of these studies various tissues were tested as substrates primarily to explore the possibility that the receptor-destroying enzyme was a collagenase. An actively desquamating effect on intestinal mucosa from rabbits and guinea-pigs was the only unequivocal result from these tests. It soon became evident that this action was not a function of the receptor-destroying enzyme but the possible relationship of such *in vitro* desquamation to the pathogenesis of human cholera seemed to justify an independent investigation of the phenomenon.

The findings to be reported are of a preliminary character but they are sufficient to indicate that the phenomenon is a complex one dependent first on autolytic processes in the bowel wall and secondly on two enzymes one or both of which may be present in vibrio culture filtrates. The first is a well-defined mucinase neutralizable by immune serum, the second can be recognized by its action on thin sections of guinea-pig tissues.

The mucinase is of particular interest since it is without action on the hyaluronic acid mucins.

MATERIALS AND METHODS.

The strains of *V. cholerae* used were obtained from Professor S. D. Rubbo (4Z) and from Dr. F. J. Wheatland of the Commonwealth Serum Laboratories (Kasauli 58, 59 [Inaba] and 52, 63 [Ogawa], Shanghai 44). The Kasauli strains were isolated in 1945.

Practically all the work has been done concomitantly with studies on the receptor-destroying enzyme (RDE) and the methods used for preparation of filtrates are identical with those described in another paper in this issue (Burnet and Stone, 1947). For most work Seitz filtrates of the fluid expressed from 16-hour cultures in soft agar were used. Allantoic fluid cultures and eluates from red blood cells were also used in some experiments.

Tests on segments of intestine were carried out as follows: Guinea-pigs that had been fasted for 24 hours were killed by a blow on the head. The abdomen was opened and the lower 18 inches of ileum removed. The contents were, if necessary, squeezed out gently and a slender glass rod passed down the lumen. The distal end was then tied to a knob at the end of the rod and the whole segment inverted. The gut was then washed and placed in Ringer solution containing excess (0·1 p.c.) $CaCl_2$. The descending colon from about two inches above the anus to the splenic flexure was also removed and the faecal pellets extruded by manipulation with the fingers. It was then inverted and washed in the same fashion.

Serial dilutions of materials to be tested were made up in Ringer + Ca solution (CaR) and in addition a series of negative (CaR) and positive (1:20 dilution of an active filtrate) controls were included. Segments of about 6–7 mm. length were now cut from ileum or colon, as required, starting from the upper end. As they were cut the segments were added to the tubes in a

[1] This work was aided by a grant from the National Health and Medical Research Council.

definite order. With say six rows of experimental tubes eight in each and a seventh row of alternating positive and negative tubes, the first seven segments cut would be placed in a vertical row comprising the first tube of each experimental and the control series, the next seven would occupy the second tubes and so on. In this way positive and negative controls were available to allow for the changing susceptibility of different regions of the gut.

The racks were placed in a 37° C. incubator for three hours. With ileum segments, readings were made immediately, but descending colon segments were allowed to remain overnight at room temperature before being examined. The readings were made by shaking the racks vigorously for a short period, then examining each tube separately, suitably illuminated against a dark background. The assessment of results was wholly on the appearance of the material liberated into the medium.

EXPERIMENTAL RESULTS.

The General Character of the Desquamation Phenomenon.

When the standard tests set up as described above with segments of guinea-pig ileum are examined after three hours at 37° C. the usual appearances are as follows: The negative control tubes will show at most a few largish flakes of liberated epithelium, while the positive controls are turbid with fine cellular flakes and discrete cells.

With an average active filtrate, dilutions up to 1:40 will show a dense turbidity with a considerable proportion of isolated cells as well as small epithelial fragments. Visible desquamation beyond that of the negative controls extends to a dilution of about 1:320, the fragments becoming larger and fewer with dilution. The endpoint is not sharp and in practice must always be assessed in terms of the corresponding negative controls. If, as is usual, there is no more than a very few (<10) flakes in the control tubes, a "+" endpoint reaction shows sufficient fragments of epithelium to give the impression of a general turbidity when gently shaken. If the control segments show excessive autolysis the endpoint has necessarily to be taken as a turbidity easily recognizable as denser than any of the controls.

When descending colon segments are used the appearances are different. After three hours at 37° C. there is very little change evident but after leaving the rack overnight at room temperature one finds on shaking that desquamation has been produced to almost the same titre as is observed after three hours with ileum segments. The unaffected segments are characteristically swollen and "fluffy" but the exudate is firmly held and is not liberated into the fluid by moderate shaking. In the presence of effective concentrations of the enzyme the supernatant fluid is turbid and the segments "clean." It is obvious that the main effect of the enzyme has been to dissolve or disaggregate the fluffy exudate.

The effects described are almost absent if the active filtrate is adjusted to pH 6 and dilutions made in acetate buffered saline at this pH. The agar filtrates used were all on the alkaline side of neutrality, usually between pH 7·5 and 8·0 and contained a considerable amount of inorganic phosphate. As carried out the tests were made at a reaction close to pH 7·6. Specific tests made in phosphate buffers at 6, 7 and 8 and in acetate and borate buffers with added Ca (M/100) showed very little difference in the results at pH 7 and 8 irrespective of the presence or absence of Ca. At pH 6 there was practically no action. The presence of Ca seemed to favour a "clean" control and was used for this reason. A disadvantage was the appearance of a turbidity due to calcium phosphate in the lowest dilutions of agar filtrates.

Protection of the "Desquamating Enzyme" of V. cholerae.

The standard method of producing active material is by growth on 1 p.c. agar plates for 16 hours followed by expression and Seitz filtration of the agar fluid. The type of result obtained in titrating such an "agar filtrate" is exemplified in Table 1 which shows the result of duplicate titrations of a batch prepared from strain 4Z (Batch 8–1).

TABLE 1.

Titration of a V. cholerae *filtrate on segments of guinea-pig ileum.*

	Filtrate dilutions.								
	20	40	80	160	320	640	1,280	2,560	Titre.
Ileum I	++	++	++	++	±±	+	±	—	640
Ileum II	±±	++	±±	±±	±±	±	—	—	480
Controls I	+	++	—	++	—	±±	—	±±	
Controls II	—	++	—	±±	—	±±	—	±±	

In each row of controls are alternative negative and positive (1:20 dilution of an active filtrate) tubes.

++ Active desquamation with fine cellular opacity ±±, +, ± diminishing amounts of desquamated material.

Using similar methods of preparation and titration some representative titres of cholera strain filtrates are shown in Table 2 along with results from some other organisms.

TABLE 2.

Activity of filtrates of various organisms.

	Desquamating titre.			
	Ileum.		Descending colon.	
V. cholerae 4Z	640	240	256	128
V. cholerae Inaba 58	640	960	64	32
V. cholerae Inaba (dialysed)	320	250		
V. cholerae Ogawa 62	64			
Salm. typhi murium	6			
Salm. derby	8			
Shigella flexneri	8			
Shigella sonnei	8			

The minor effect shown by filtrates from a variety of other organisms has a different appearance and is never complete. It is not significantly reduced by boiling nor is it inhibited by immune sera active against cholera filtrates.

Dialysis of active cholera preparations produces only a small diminution in activity. This has been confirmed on several occasions.

Differences in Reactivity of Ileum and Descending Colon.

The general diminution in sensitivity of segments as one passes successively downwards from jejunum to the descending colon has already been indicated. With jejunal segments there is a clearly visible effect but this is rapidly obscured by the occurrence of desquamation in the controls. The gross differences between ileum and colon have been described but it should be stressed that in addition there is a clear gradient of susceptibility to desquamation within each section of the bowel. It is for this reason that positive and negative controls representative of each portion of the gut used are included in all tests.

There is a further apparently qualitative difference between ileum and descending colon, the possible significance of which is referred to later. Active agar filtrates act to more or less equivalent dilutions on ileum and descending colon segments provided an appropriately longer time is allowed for action on the colon segments. Preparations from allantoic fluid cultures of *V. cholerae* with an equally high RDE content, however, produce desquamation of ileum segments to a relatively high titre but have no significant effect on the colon. The action on the ileum appears to be quite similar to that of agar filtrates and, as will be shown in a following section, it is specifically inhibited by an immune serum.

The same inability to affect the descending colon is shown by eluates from red cells prepared primarily to concentrate the receptor-destroying enzyme. As far as the relatively inaccurate method of titration allows one to estimate, the degree of concentration of desquamating agent obtained by this method is only about one-fourth of that obtained of RDE but there is no question of the reality of this adsorption and elution of the desquamating agent.

Thermal Inactivation.

The agar filtrates as used have normally an alkaline reaction pH 7·5–8. When such filtrates are heated to 56° C. for 30 minutes the greater part of their activity is lost. When tested on ileum segments desquamation beyond the controls will only be seen to a dilution of 10–20. On descending colon there is an even more marked reduction, activity at 1:4 being the highest figure noted. When adjusted to pH 6·2 the same destruction was evident, in this case with no residual effect even at a dilution of 1:2.

In a previous paper we have described the effect of calcium ions in raising the temperature of inactivation of the receptor-destroying enzyme in the same filtrates. A number of preparations made primarily in the course of studies on this phenomenon were also tested for their desquamating activity. It was found that filtrates heated undiluted at 55° C. for 30 minutes at various reactions with or without added calcium were all either totally inert or gave the low titre changes that are also seen with boiled material. Inactivation was also practically complete when untreated filtrates (pH 7·6) were heated at 50° C. or 52° C. Dialysed material was less readily inactivated, about 25 p.c. of activity remaining after 30 minutes at 50° C. and a significant activity at 52·5° C. for the same time. Filtrates diluted 1:20 in buffered calcium saline before heating and tested only in that dilution showed definite activity after 57° C. but none after 60° C. for 30 minutes.

These experiments showed that while the activity was destroyed by heating to 60° C. for 30 minutes under all conditions, it might be destroyed at considerably lower temperatures depending on the medium in which it was suspended.

When most filtrates were boiled they retained a slight action on ileum segments, giving rise to an opacity in the fluid and slightly increased desquamation rather characteristically greater in 1:4 than 1:2 dilutions. This action is not neutralized by serum and appears to be similar to that observed with Salmonella or Shigella filtrates. The existence of this minor heat-stable effect not extending beyond a 1:10 dilution made it difficult to obtain clear-cut results in thermal inactivation tests and it was not thought expedient to study the effect of the medium on the inactivation process in any detail. The evidence is sufficient to show that the essential component of the reaction is destroyed in 30 minutes at 55–60° C.

Neutralization of Desquamating Effect by Immune Sera.

Several rabbit sera from animals injected twice with various *V. cholerae* preparations showed no power to inhibit the reaction so three rabbits which had already survived a large dose of filtrate given in tests for lethal action were submitted to intensive immunization. Each received 0·5 ml. of crude filtrate intravenously twice a week for four weeks. They were bled ten days after the last inoculation. The sera were heated at 56° C. for 30 minutes and stored in the cold.

All three sera had a well-marked inhibitory effect not shown with normal rabbit serum. The standard form of test used was to mix falling dilutions of serum with an equal volume of a 1: 10 dilution of an active agar filtrate and then add a segment of ileum or descending colon to each tube. The immune sera regularly inhibited the reaction to an easily recognized extent and it was possible to show that the strength of the sera was in the order 147 <149 <148. The titres obtained for R147 were 120–160 in three tests. R148, 320–1,280 in eight tests. R149, 200–640 in six tests. Over 20 tests with six normal rabbit sera and a number of sera from inadequately immunized rabbits, all gave no evidence of inhibition of the reaction from final dilutions of 1: 20 onward.

Smaller numbers of tests showed that the activity of allantoic fluid and red cell eluate preparations could be neutralized by these immune sera but not by normal rabbit serum.

Since the endotoxin of the cholera vibrio is usually regarded as being identical with the polysaccharide-lipid complex that makes up the O antigen, anti-endotoxin should be absorbed by washed vibrios although we have been unable to find any account of such experiments. Sera active against the desquamating enzyme were therefore absorbed with large quantities of washed vibrios in the living state. The procedure was to mix 2·5 ml. of serum diluted 1:10 in saline with 0·25 ml. of packed vibrios scraped from 16-hour cultures in soft agar and washed twice with saline. The possible liberation of desquamating enzyme by the vibrios was controlled by using a normal rabbit serum similarly treated and testing its action on gut segments in the absence of any added enzyme. A trivial effect only was observed.

Table 3 gives the result of this experiment in abbreviated form. No evidence was obtained that the activity of the serum against the desquamating action was reduced by absorption with vibrios, although the agglutinin titre was reduced to 2 p.c. of that of the original.

The low titre action of boiled agar filtrates from vibrio cultures in accelerating desquamation of ileum segments was not neutralized by immune sera.

TABLE 3.

To demonstrate action of immune serum and failure to absorb antibody from serum with washed vibrios.

	Serum dilutions.							
Serum.	20	40	80	160	320	640	1,280	2,560
Unabsorbed								
N.R.S.	++	++	++	++	++	++	++	++
R 148	—	—	—	—	—	+	++	++
R 149	—	—	—	—	±	±	++	++
Absorbed								
R 148	±	±	±	±	±	±	+	++
R 149	±	—	±	±	±	±	++	++
N.R.S. (no enzyme)	±	±	±	—	—	±	±	—
Enzyme control	++		++		++		++	
Saline control		±		—		—		—

Histological Changes.

No detailed histological investigations have been made but many segments at different stages of the process have been examined under a binocular dissecting microscope and haematoxylin-eosin stained sections have been made from segments at various stages. The process appears to be essentially similar in both ileum and descending colon. In both, the major histological change is due to spontaneous autolytic and desquamative processes, the essential effect of the vibrio filtrate being to loosen the desquamated fragments from their attachment and allow their dispersion in the medium.

The process is seen most clearly with segments of descending colon. When the inverted segments are left in any saline medium for some hours, the mucosa swells and the superficial epithelium is broken up into flakes which are held in position by the large amount of tenacious mucous exuding from the glands of Lieberkuhn. When active enzyme preparations are added the mucin is dissolved (? depolymerized) and the superficial epithelium is completely shed leaving a ragged surface. Sections show that desquamation has extended down the crypts to a variable extent and that most of the goblet cells are discharged. Unless special care is taken not much difference is to be seen between sections exposed only to the medium and those on which the enzyme has acted, since most of the loosely held fragments are lost in the course of manipulation.

With small intestine segments from the guinea-pig the process can be well seen under the dissecting microscope. After three hours in buffered (pH 7–8) saline at 37° C. the leaf-like villi are swollen and packed closely together, fluid accumulating beneath the epithelial layer. In the presence of an active concentration of enzyme the appearance is identical so long as the segment is not exposed to movement or trauma. If now the two segments are tested by attempting to remove some of the epithelium with a needle point under a tenfold magnification very characteristic differences are observed. With the control segment small portions of epithelium can be readily dislodged but as they are drawn away, threads of mucus holding them to the deeper layers become visible. As the process is continued a mesh of mucin holding broken fragments of epithelium is produced. In the presence of the cholera enzyme a very light touch with the needle is sufficient to dislodge the epithelium covering a villus, which lifts off in large flakes. There are no mucin strings and the dislodged fragments fall immediately to the floor of the dish.

If control and treated segments are vigorously shaken the epithelium is almost completely removed from the latter, while the control develops only a rather ragged fuzzy outline with a few liberated flakes. Segments completely desquamated will usually show a series of parallel or herringbone brownish lines representing the blood in the exposed capillary network of each villus. Histological sections fully confirm the interpretation gained from observations under the dissecting microscope provided one allows for the inevitable further damage associated with unavoidable manipulations. The dominant effect of the treatment is to remove the viscous material which holds the epithelium on to the oedematous core of the villus. The impression from these observations was irresistible that the main action was due to a mucinase which rapidly converted the viscous strings of mucin to some non-viscous derivative.

Mucinase Activity.

The main difficulty in establishing directly that the enzyme mainly responsible for desquamation was a mucinase was the absence of suitable substrate material. Extracts from oedematous bowel wall were unsatisfactory and commercial gastric mucin could not be brought into a suitable form for test. Since we were primarily interested in intestinal mucin the most direct method seemed to be to use mucin as secreted into the lumen of the ileum. Fasted guinea-pigs were anaesthetized with ether and the lower portion of the ileum exposed by laporatomy.

TABLE 4.

Mucinase tests with guinea-pig intestinal fluid as substrate.

	Dilution.							
Preparation.	5	10	20	40	80	160	320	640
Ag F. 4Z 1	—	—	—	—	tr	±	+	+
Ag F. 4Z 2	—	—	—	—	—	—	—	±
Ag F. 4Z H 55°	+	+	+	+	+	+	+	+
All fluid. Inaba	—	—	—	±	+	+	+	+

+ Pellicle formation when mixture dropped into acid alcohol after 1 hour at 37° C.

Ag F. Filtrate from 16-hour soft agar culture.

All Fluid. Allantoic fluid culture (16 hours) centrifuged and treated with 1:10,000 merthiolate.

After the contents had been "milked" out as completely as possible a length of about 20 cm. was tied off and sufficient M/15 phosphate buffer pH 7·4 injected to distend the segment (8–10 ml.). The abdominal wall was then closed and the animals kept under sodium amytal or morphine sedation for two to three hours before being killed. The contents of the lumen were then withdrawn by syringe and when the preliminary emptying of the bowel had been satisfactory, the fluid was clear, usually yellowish, and very viscous. This fluid was used untreated as substrate for mucinase tests.

TABLE 5.

Neutralization of mucinase by immune serum.

	Serum dilutions.								Controls.		
Sera.	5	10	20	40	80	160	320	640	S/5	Saline.	Ag F/10
R 161 Normal	—	—	—	—	—	—	—	—	+	+	—
R 148-3 Immune	+	+	+	+	±	—	—	—	+	+	—

Serum dilutions made in active agar filtrate (Ag F. 4Z 2) diluted 1: 10 in buffered Ca saline. S/5 1: 5 dilution of serum without added enzyme.

Two methods were developed to show the destruction of mucin by the enzyme without having recourse to viscosimetric measurements. The first was to mix with the mucin solution about 0·5 p.c. of red cells. When the tubes were shaken after reaction had taken place there was an easily visible difference between the free redispersion of the cells where the mucin had been destroyed, and the tenacious coil of red cells and mucin raised in the unchanged mixtures. The second was to let a drop from each mixture fall into acid alcohol (1 p.c. HCl in 70 p.c. alcohol). If unmodified mucin is present the drop maintains its form owing to the development of a tenacious pellicle: when the mucin is destroyed (? depolymerized) the drop spreads out and disappears apart from a cloudy precipitate. Both tests give equivalent readings which also agree with the changes in viscosity which become evident when removing samples with a capillary pipette. The presence of serum in mixtures somewhat weakens the pellicle but there is no difficulty in distinguishing between positive and negative results.

Once this material was available, there was no difficulty in establishing (1) that all preparations containing desquamating enzyme contained mucinase, (2) that the same antiserum had an equivalent inactivating effect on the desquamating and mucinase reactions, and (3) that the mucinase was similarly thermolabile. Tables 4 and 5 show the results from representative experiments on these points.

Evidence for the Existence of an Additional Enzyme in Agar Filtrates of V. cholerae.

We have already noted that allantoic fluid preparations are inactive on segments of descending colon, although showing activity on ileum segments approximately equal to that of agar filtrates. Further evidence of the existence of an additional active component in the agar filtrates has been obtained by two different methods. Examination of the process under the dissecting microscope shows that with allantoic fluid material desquamation of the villous epithelium takes place characteristically in long "troughs" which may be up to 2 mm. in length and which remain coherent on gentle manipulation. With agar filtrates the "troughs" are shorter and more fragile. By desquamating epithelium with allantoic material and washing the fragments gently with saline a suspension could be obtained which could be used to determine the existence of a secondary disintegrating effect. Preliminary experiments indicate a well-marked breaking down of these flakes by active agar filtrates but not by boiled material nor by allantoic fluid filtrates alone or mixed with boiled agar filtrates. Another approach has been by the use of frozen sections 25μ thick from guinea-pig liver. If these are exposed at 37° C. for 16 hours with penicillin as a bacteriostatic agent gross disintegration occurs, with active agar filtrates up to a dilution of 1:10 or 1:20, while controls in saline show no visible effect. Again, heated agar filtrates, or unheated allantoic fluid preparations show no effect. Study of this reaction is actively in progress in collaboration with Dr. J. Perry and details will be reserved for another publication.

DISCUSSION.

The general impression derived from a survey of the literature on toxic products of *V. cholerae* is that the only clearly defined toxic agent is an endotoxin of the kind characterizing most of the Gram-negative intestinal pathogens. According to Gallut and Grabar (1945), it is a polysaccharide-lipid complex

liberated in polymerized form on autolysis of the organisms. By analogy with other similar bacteria this endotoxin should be antigenically equivalent to the O-agglutinogen but we have found no specific experimental evidence on this point. The appearance of soluble products with acute toxicity for mice and guinea-pigs has been shown by many authors to be coincident with lysis of the vibrios and it is generally agreed that no true soluble exotoxins are formed. The most recent American work on the subject differs from the classical viewpoint only in ascribing the essential toxicity to the lipid component of the antigenic complex, Burrows (1946).

As far as we can gather none of the three agents in cholera vibrio filtrates with which we have been concerned has previously been studied as a possible element playing a part in the pathogenesis of cholera. It is highly suggestive however that this organism produces free enzymes active against cell components or products in much higher concentration than any of the other intestinal bacteria we have tested; and it is difficult to believe that one or more of them is not in some way directly related to the characteristic pathogenicity of the vibrio. The significance of the receptor-destroying enzyme will not be clear until its substrate is determined. The mucinase described in this paper can rapidly destroy the viscosity and hence the mechanical protective and lubricating properties of intestinal mucus. Experiments in progress show that this action can take place in isolated gut segments in the living animal and if the enzyme, as seems likely, is produced in large amount in the bowel of a cholera patient, it might well play a major part in facilitating desquamation of the intestinal epithelium. The third agent, on which very little work has so far been done, by breaking down some presumed components of the cement substance between cells would also favour the desquamating process.

The desquamation of epithelium from segments of gut *in vitro* is a highly artificial reaction and can certainly not be taken as indicating that similar results would be produced by the same agents under appropriate circumstances *in vivo*. The reaction is absolutely dependent on the occurrence of autolytic and oedematous changes and can be prevented by maintaining the system at pH 6 when such changes are greatly diminished. We have given evidence that both a mucinase and a tissue-disintegrating enzyme are concerned in the reaction. The receptor-destroying enzyme appears to have no rôle and it may be added here that active hyaluronidase from mouse testis and from a staphylococcus culture were completely without effect on ileum.

The production by *V. cholerae* of a mucinase which is active against intestinal mucin but not against hyaluronic acid is of considerable interest. There appears to have been very limited biochemical work done on the mucins produced by the glands and mucous membranes and judging from the stimulus to work on the connective tissue mucins that was provided by the recognition of the existence and importance of hyaluronidases, the use of vibrio enzyme may come to be a valuable help in the chemical study of this field. The fact that a specifically-neutralizing immune rabbit serum can be produced considerably increases the value of the enzyme as a reagent.

SUMMARY.

Filtrates from *Vibrio cholerae* cultures are capable of producing desquamation of the intestinal epithelium *in vitro*.

There is a well-marked gradient of diminishing susceptibility to desquamation from the jejunum to the descending colon.

Histological and other preliminary evidence suggests that the principal agent concerned is a mucinase.

Intestinal mucin is rapidly dissolved by active filtrates, the effect paralleling the desquamation reaction; both are similarly neutralized by rabbit immune serum.

Evidence is given for the existence of another enzyme concerned with breaking down the cement substance between cells.

The possibility that these enzymes may play a part in the pathogenesis of cholera is discussed.

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THE RECEPTOR-DESTROYING ENZYME OF *V. CHOLERAE*

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In previous papers we have described (Burnet, McCrea and Stone, 1946) the activity of filtrates from *V. cholerae* in removing "virus receptors" from red cells. Cells so treated also develop the condition of panagglutinability described by Thomson (1926) and Friedenrich (1928) and evidence has been given that both effects are due to the action of an enzyme which for convenience we refer to as receptor-destroying enzyme, RDE. In the second paper (Stone, 1947) of the series, it was shown that except for minor details the action of viruses of the influenza group on the red-cell surface was essentially the same as that of RDE.

The present paper reports further work which has been directed mainly toward determining the optimal conditions for the production of enzyme by the vibrio, and for the preparation of purified material by elution from red cells.

EXPERIMENTAL.

The Production of RDE.

In the work reported previously, RDE was prepared by the growth of a suitable *V. cholerae* strain in nutrient broth for 16–20 hours. Filtrates from such cultures gave titres of the order 160–240. Growth in simple peptone water, though producing similar degrees of turbidity, gave only very small yields (<30).

Much higher titre filtrates could be obtained by the method used by one of us in 1930 for the production of staphylococcal toxin (Burnet, 1930). Plates of weak (1 p.c.) nutrient agar were inoculated with a few drops of broth culture of the desired strain and incubated for 16 hours at 37° C. The surface growth was scraped off with a glass rod and the agar removed into a gauze bag inside the top of a Buchner funnel. Usually a batch of 20 Petri dishes was used. The gauze bag was tied and the fluid pressed out by a weighted cylindrical jar fitting closely into the top of the Buchner funnel. The expressed fluid was centrifuged if necessary and passed through a Seitz filter. These "agar filtrates" had a pH between 7·5 and 8·5. Their RDE titres with suitable strains ranged from 320 to 2,000, and the method has been used as a routine both for the production of RDE and also for the desquamating enzyme described elsewhere.

A second effective method is growth of a suitable strain in the allantoic cavity of 12-day chick embryos. Death occurs with a small inoculum in approximately 24 hours and fluid harvested at this time and centrifuged gave titres of almost exactly the same range, 400–2,000, as those obtained with agar filtrates. A disadvantage of the method is the great loss of RDE activity which follows passage through a Seitz filter. For comparison with the results given in Stone's 1947 paper it may be noted that dried ammonium sulphate precipitated preparations dissolved at a strength of 50 mg. per ml. had titres ranging from 1,640 to 3,200.

Six strains of cholera vibrio have been tested with the results shown in Table 1.

TABLE 1.

Strains.	RDE titres. Agar filtrates.	Allantoic filtrates.
V. cholerae 4Z	1,200, 1,600, 2,000	300, 470, 1,200
Kasauli strains		
Inaba 58	2,000	1,400, 1,600, 2,000
Inaba 59	500	
Ogawa 62	2,560	700, 1,000, 1,280
Ogawa 63	160	
V. Shanghai 44	30	

[1]This work was carried out under a grant from the National Health and Medical Research Council.

Three of the strains, including one known Inaba and one Ogawa sub-type, gave high titre material. Three others gave only low titre enzyme. The great majority of experiments to be described were done with agar filtrates from strain 4Z.

Agar filtrates were prepared from cultures of the following organisms: *Shigella flexneri* (two strains), *Sh. sonnei*, *Sh. shiga*; *Salmonella typhi murium*, *S. derby*; *Esch. coli*. None showed any activity at a dilution of 1:2 or higher.

If incubation of the nutrient agar plates inoculated with *V. cholerae* was extended beyond 16 hours, results were highly variable. With some batches of agar, there was a marked fall at 24 hours and complete absence of RDE in filtrates from 48-hour cultures. With others only a slight reduction in titre occurred on further incubation.

Method of Titration.

It was shown earlier that the activity of RDE is fully developed only in the presence of free calcium ions and that a concentration of about 0·1 p.c. $CaCl_2$ in normal saline was effective (Stone, 1947). It seemed desirable to use a saline buffered about neutrality and for routine use a solution was prepared containing 1·00 gm. $CaCl_2$, 9·00 gm. NaCl, 1·203 gm. H_3BO_3 and 0·052 gm. $Na_2B_4O_7.10\ H_2O$ per litre of distilled water. As used the pH lay between 7·1 and 7·3. Subsequent work indicated that a slightly more acid reaction would have given higher titres, but it has seemed desirable to retain the above solution as the standard diluent.

In designing a method of titration, the main requirement was speed and convenience with a reasonable degree of accuracy. Three main possibilities were available.

1. To allow the action of graded dilutions of RDE on a standard amount of red cells at 37° C., then to chill and wash the cells and finally test with a full series of viruses to find to what level in the gradient receptors had been removed.

2. To add a standard amount of red cells to graded RDE dilutions, allow interaction at 37° C. to take place for a convenient period and then to test the cells with a suitable dose of a single virus.

3. To proceed as in 2, but to test the exposed cells with a suitable normal serum in the cold. Cells sufficiently affected by the enzyme will be agglutinated.

All three methods have been used for specific purposes, but the second has proved by far the most convenient, and after testing out a number of variants the following technique has been adopted.

Serial dilutions of the enzyme-containing material are made in the buffered calcium saline. 0·25 ml. volumes are used in test tubes of 1·0 cm. internal diameter. A similar volume of 1 p.c. washed fowl cells in normal saline is added and the tubes left for 30 minutes in a water bath at 37° C. Then one drop (0·025 ml.) of a dilution of virus, Melbourne M, containing 100 agglutinating doses as normally measured is added to each tube, giving ten agglutinating doses. The racks are removed for a few seconds to be shaken thoroughly and are then replaced. Readings are made 30 minutes later, by which time the cells have just settled to the bottom and agglutinated cells have not begun to slip. The endpoint taken is the usual "+" degree of partial agglutination.

The Influence of Ca Ions on the Enzyme.

Owing to the relatively high content of inorganic phosphate in crude filtrates the influence of Ca ions is best demonstrated with dialysed material. If the results with normal saline (0·9 p.c. NaCl solution) as diluent are used as a standard, it is found that higher titres are regularly obtained when small concentrations of $CaCl_2$ are present in the diluent. The exact amount is unimportant, similar titres being obtained with concentrations between M/50 and M/400 of $CaCl_2$.

TABLE 2.

Influence of composition of diluent on observed titre of RDE.

pH.	Ca.	Other constituents.	Titre.
5·6	M/100	Acetate	480
5·9	M/100	Acetate	1,000
6·5	H/100	Acetate	1,280
7·0	M/100	Borate	1,000
7·2	—	Normal saline	280
7·2	M/400	Normal saline	1,600
7·2	M/200	Normal saline	1,600
7·2	M/100	Normal saline	1,400
7·2	M/50	Normal saline	1,280

Relatively little reduction of titre is obtained when fluoride or phosphate is added to the normal saline diluting fluid but a sharp reduction occurs with equivalent concentrations of oxalate, citrate or sodium hexametaphosphate.

In the presence of M/100 Ca the optimal pH is about 6·5 but there is little difference between pH 6·0 and 7·5. At reactions more alkaline than pH 8 there is a marked decrease in titre.

These points are exemplified in the series of tests made with dialysed agar filtrate which are shown in Tables 2 and 3.

TABLE 3.

Effect of Ca and Ca de-ionizing agents on activity of RDE.

Diluent.	RDE titre.
Normal saline	320
M/100 Ca Cl_2	1,200
M/30 Sodium citrate	5
M/20 Pot. oxalate	15
M/10 Sodium fluoride	240
M/30 Sodium phosphate	160
M/60 Sodium hexametaphosphate	<5

All solutions made in normal saline and adjusted to pH 7·0–7·2. An equal volume of red cells in normal saline is added bringing final concentrations to half those shown.

Thermal Inactivation of RDE.

In preliminary tests it was found that when agar filtrate preparations were heated for 30 minutes at 56° C. all activity was lost but that if the material was diluted 1:10 or 1:20 in buffered calcium saline very little inactivation occurred under the same conditions.

Detailed investigation showed that undiluted material was partially inactivated at 50° C. and completely at 52·5° C. in 30 minutes, while the same material diluted in calcium saline required a temperature of 60° C. for complete inactivation (Fig. 1). Further analysis of the effect using RDE in the form of eluates in Ca saline from red cells gave confusing results, apparently due to Ca retained by the enzyme even in the presence of phosphate, but clear cut results were obtained when dialysed agar filtrate material was used.

Table 4 shows the residual activity after dialysed RDE of titre 2,400 had been heated for 30 minutes at 55° C. when diluted 1:20 in various diluents.

TABLE 4.

Thermal inactivation of RDE in various diluents.

pH.	Diluent.	Titre.	pH.	Diluent.	Titre.
5·6	M/100 Ca + Saline	2,000	7·2	Ca M/50 Saline	1,000
5·9	,,	2,000	7·2	M/100	1,000
6·5	,,	1,800	7·2	M/200	500
7·0	,,	1,280	7·2	M/400	70
8·5	,,	20	7·2	Normal saline	<20

Original titre 2,400. All dilutions 1:20 heated 55° C. for 30 minutes.

All experiments with dialysed material have shown complete inactivation at 55° C. in 30 minutes unless Ca has been added to the diluting fluid. The full protective effect is obtained with M/100 Ca but not with smaller amounts. The protective effect of Ca is evident at all pH ranges from 5·6 to 8·5 but becomes much less effective on the alkaline side.

Absence of Antigenicity of RDE.

Much effort has been expended in trying to obtain an antiserum active against RDE. Rabbits and guinea-pigs have been inoculated intravenously or intracardially with crude

filtrates, eluates and treated cells without in any instance providing serum which could inhibit the action of small amounts of RDE. Tests were made by two techniques. In the first the sera were absorbed with cells treated with RDE to remove natural agglutinin for such cells. Serial dilutions of serum from 1:5 onwards were then prepared and a standard volume of RDE containing five effective doses added to each tube. After standing for 30 minutes one drop of 5 p.c. red cells was added and thereafter the ordinary procedure for RDE titrations was followed. Controls of each serum with RDE-treated cells alone were included. The second method was to detect change in the cells due to RDE action by the agglutinating effect of the serum itself. A constant amount of each serum normal and after immunization was added to series of RDE dilutions. Red cells were then added and after one hour at 37° C. the racks were shaken and the cells allowed to settle in the refrigerator. Both methods gave completely negative results.

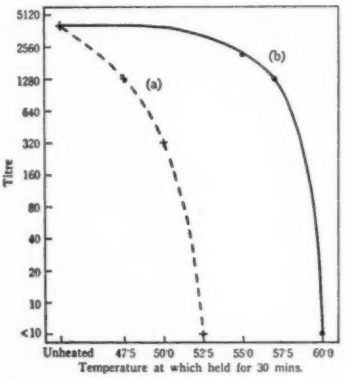

Fig. 1. Thermal inactivation of RDE.
(a) Filtrate diluted in normal saline.
(b) Filtrate diluted in saline with 0·1 p.c. $CaCl_2$.

Preparation of Purified RDE by Elution from Red Cells.

The possibility of producing material by adsorption to red cells in the cold and subsequent elution at 37° C. has been described in the earlier papers of this series. Further experiments have been carried out to determine the optimal conditions for this procedure, human red cells of group O being used throughout.

No adsorption of the enzyme can be demonstrated at temperatures of 20° C. or 37° C. but if reagents are chilled to 0° C. and the treated cells washed with cold saline, adsorption can be readily demonstrated and the enzyme subsequently eluted.

Adsorption is favoured by an alkaline reaction and is not significantly influenced by the presence or absence of Ca ions. Investigation of the optimal conditions for adsorption was largely influenced by the desirability of producing relatively large amounts of high titre eluate for chemical investigations. Most of the work was, therefore, carried out with preparations made by adding an excess of Ca to crude unfiltered agar extract, heating for 30 minutes at pH 6 to destroy vibrios and centrifuging to remove calcium phosphate and bacterial bodies. The final supernatant fluid is opalescent and may deposit a loose phosphate precipitate on standing.

Table 5 gives a representative series of results showing the activity remaining after fluids adjusted to various pH levels had been absorbed with human cells in the cold and the percentage of total activity recovered in the eluate.

TABLE 5.

Influence of pH on absorption of enzyme by red cells.

pH.	Supernatant.	Eluates.
6·0–6·2	100	5–8
7·0–7·3	80	20
8·0–8·5	50, 30 (26)	41, 20 (25)
8·6–8·9	25, 20	35, 40
9·0–9·8	12, 7, 7, 6, 3 (13)	90, 50, 40, 35, 37, 37 (33)

(Values in brackets with excess phosphate: others excess Ca ion)
Percentage of initial titre shown.

It will be seen that there is a regular increase in the completeness of absorption with increasing alkalinity. A comparative experiment with material adjusted to pH 9·2, 9·5, and 9·8 indicated that no consistent improvement occurred above 9·2, all supernatant fluids giving approximately the same values. For production of eluates, therefore, the crude material is adjusted to pH 9·2–9·4, using thymol blue as indicator.

It is apparently almost immaterial what fluid is used to elute the enzyme from red cells provided adsorption occurs at an adequately high level of alkalinity. In view of the fact that destruction of receptors is most active at a slightly acid reaction in the presence of calcium, we have normally used our standard calcium saline buffered with borate at pH 7·0

or acetate saline at pH 6·0 but comparative tests showed no significant differences in yield with phosphate buffers at pH's 6, 7 and 8. Since, however, the phosphate solutions produced much more haemolysis than those with an excess of Ca, the latter are always used.

Very little time is required to allow maximal adsorption at 0° C. Table 6 gives the results of an experiment in which chilled cells and crude enzyme (pH 9·2 with excess Ca) were mixed and centrifuged after standing for various periods at 0° C.; the crude material had a titre of 3,200. Five ml. were used in each tube with 0·25 ml. of packed human red blood cells. The tube labelled zero time was thoroughly mixed and placed immediately in the centrifuge, spinning being completed in two minutes. All manipulations including the centrifugation were carried out in the cold room—0° C.

TABLE 6.

Titres of supernatant fluids after contact with cells at 0° C.

Time of contact.	Titre.
0 minutes	200
2 minutes	160
5 minutes	160
10 minutes	480
20 minutes	640
30 minutes	1,600
Unabsorbed	3,200

It will be seen that almost maximal absorption is obtained within less than two minutes, while after five minutes reliberation commences and has reached 50 p.c. by 30 minutes. As would be expected from this result, elution is also a rapid process at 37° C. A pool of the cells treated in the above experiment and washed once with Ca saline at 7·0 was divided into six portions, three being eluted into Ca saline at pH 7 (borate) and three into Ca saline at pH 6 (acetate). The tubes were centrifuged at 2, 15 and 60 minutes and the supernatant fluids titrated.

TABLE 7.

Rate of elution from red-cells.

pH	Time of elution.	Titre of eluate.
6·0	2 minutes	6,000
	15 minutes	6,000
	30 minutes	7,000
7·0	2 minutes	7.000
	15 minutes	7,000
	30 minutes	7,000

Since the nominal concentration in this experiment was fourfold only about half the theoretical amount absorbed was recovered in the eluates. A further exposure of cells initially treated for two minutes to the same volume of fresh saline gave a secondary eluate with an RDE titre of 2,000.

Under suitable circumstances a yield of up to 80 p.c. can be obtained and there is no evidence that any of the enzyme is used up in the process of adsorption, elution and receptor destruction. Two experiments showing this may be mentioned briefly. In the first, 5 ml. of crude RDE (heated in the presence of Ca and adjusted to pH 9·2) were successively treated with four lots of 0·25 ml. of packed human cells. The cells were added to chilled fluid, then the mixture placed at 37° C. for an hour and centrifuged. The cells were removed, washed and tested for completeness of receptor destruction and for the appearance of "T" agglutinability. The supernatant fluid was chilled and the same procedure repeated four times, sufficient being removed at each step to allow RDE titrations. The results showed no significant reduction in titre of RDE at any stage and all cells were completely modified apart from a trace of agglutination by SW virus in the first series.

In the second experiment after adsorption in the cold the cells were quickly washed with cold saline and then resuspended in a volume of cold buffered Ca saline equal to the original material. Elution was allowed to take place for an hour at 37° C. and the *eluate* used for the next series of similar manipulations. The final eluate was thus made up of enzyme which had been through four successive washings while adsorbed to the red cell surface. RDE titres of the original material and of the four successive eluates were respectively 1,600, 1,200, 900, 800, 800. Allowing for minor inaccuracies in titration, this represents approxi-

mately an 80 p.c. yield at each manipulation. When the enzyme is eluted into smaller volumes in order to obtain higher concentrations incidental losses are increased and the average yield is about 60 p.c. of the original.

Based on these experiments the following technique is being used for the production of eluates as an initial stage of purification.

To the crude agar extract (100 ml.) 2·5 ml. of 20 p.c. $CaCl_2$ solution are added. This produces a copious precipitate of calcium phosphates and brings the pH initially 7·6 to 8 to about 6·0. If necessary the pH is adjusted with normal NaOH or HCl to this value and the material is then heated for 40 minutes in a flask completely submerged in a water bath at 55° C. The phosphate precipitate is now flocculent and an almost clear supernatant fluid is obtained on centrifugation.

The fluid is now brought to pH 9·2 by the addition of 4 to 5 ml. of normal NaOH.

Human red cells from defibrinated blood are washed twice with normal saline. From 0·5 ml. to 1 ml. of packed cells is added to each 10 ml. of fluid in centrifuge tubes, all reagents and tubes being chilled to 0° C. As soon as the red cells have been thoroughly dispersed the tubes are rapidly centrifuged, the supernatant fluid removed and replaced by a similar volume of chilled Ca saline. After a further quick centrifugation the washing is discarded and the cells emulsified in one-fourth the original volume of calcium acetate buffered saline pH·6 and placed in a water bath at 37° C. for one hour. The eluate is then obtained by a final centrifugation. When it is desired to obtain an eluate with the least possible amount of haemolysis the time of elution is reduced to five minutes.

DISCUSSION.

This paper is concerned with the essentially practical problems of means of titrating the RDE of *V. cholerae* and of preparing concentrates by elution from red blood cells. In the course of the work some information of interest on the nature of the enzyme-cell interaction has been obtained.

In the titration method adopted the concentration of enzyme is determined which at an appropriate time and temperature will almost remove (to one plus level) the agglutinability of a certain amount of red cells by virus Melbourne. It is assumed that during the period of the test, enzyme molecules

(a) become adsorbed to receptors on the red-cell surface,
(b) act on these causing their destruction or liberation, and
(c) are then set free to pass to another receptor on the same or another cell.

The optimal conditions 37° C., slight acidity and the presence of Ca ions are those which favour the over-all efficiency of this sequence. Analysis of the various phases of the sequence shows that optimal conditions for adsorption are 0° C., high alkalinity (pH 9 or higher) with no clear evidence of any beneficial effect of Ca. Elution of the bulk of the adsorbed enzyme takes place rapidly at 37° C. and there is concomitantly a loss of receptors for the viruses at the lower end of the gradient. Complete removal of receptors, however, in most experiments requires a longer period during which further elution of enzyme also takes place.

The recognition of the sharp effect of Ca ions in protecting against thermal inactivation at 55° C., especially at acid reactions, allows a practical method of sterilizing material without filtration to serve as a starting point for purification by elution from red cells. It is possible that this protective effect of Ca may give a clue to the nature of the enzyme, but we can find no comparative data for other enzymes in this respect.

SUMMARY.

A study has been made of the conditions for production, titration and partial purification of the enzyme produced by *V. cholerae* which destroys the "virus receptors" of red blood cells.

The enzyme is produced in good yield either by growth on soft agar plates or by cultivation of the vibrio in the allantoic cavity of the chick-embryo.

A method for rapid titration of enzyme activity is described.

The enzyme is thermolabile, being completely destroyed in the absence of Ca after 30 minutes' heating at 52·5° C. In the presence of M/100 Ca the thermal inactivation point is raised to 60° C.

Adsorption of enzyme to red cells takes place rapidly in the cold, the optimal pH for the reaction being about 9·2. If such cells are washed in the cold, and the enzyme subsequently allowed to elute at 37° C., concentrated preparations free of most of the original impurities can be obtained.

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SPORADIC AND MINOR EPIDEMIC INCIDENCE OF INFLUENZA A IN VICTORIA, 1945–46

I. PHASE BEHAVIOUR OF INFLUENZA A STRAINS IN RELATION TO EPIDEMIC CHARACTERISTICS

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INTRODUCTION.

A feature of recent influenza studies both in the northern hemisphere (Andrewes and Glover, 1944; Salk, Menke and Francis, 1944) and in Australia has been the recognition of sporadic cases or limited outbreaks of true influenza during the months preceding or following a typical epidemic. Influenza A epidemics occurred in Melbourne in July, 1935, July, 1939, and May, 1942. During 1943, small numbers of both A and B infections were recognized by Beveridge and Williams (1944). No further A infection was recognized until the occurrence of the October, 1945, epidemic described by Burnet, Stone and Anderson (1946). This was shown to be due predominantly to influenza virus B but from a minority of patients the virus isolated was of A type. The persistence of the latter type in the community was indicated by the isolation of this virus from a sporadic case in February, 1946; ten weeks later a minor epidemic of influenza A broke out in Melbourne and subsequently in Geelong.

The work reported in the two present papers was concerned with the isolation and study of the A strains of 1945 and 1946. Attention was directed particularly to the O–D phase behaviour of the strains isolated (first paper) and to the "breadth" of the immunological response in different infected individuals (second paper).

MATERIALS.

Strains of Influenza A Virus.

BEL—One of the strains isolated during the 1942 epidemic in Melbourne.
CAM—A recent strain isolated from a pool of throat washings from the first three cases seen in the 1936 epidemic.
CKS—A strain derived from a pool of three throat washings in 1943 and used for the production of A type vaccine in Melbourne.
GAT—A classical strain (Smith and Andrewes, 1938).
IAN—Isolated from a sporadic case of influenza A in February, 1946.
Melbourne—A strain isolated in Melbourne in 1935.
MIL A—A strain isolated in Melbourne during October, 1945.
SW—Shope's strain 15 of swine influenza virus.
WSE—The classical strain WS adapted to chorioallantoic passage.

Strain of Influenza B Virus.

MIL B—Isolated in October, 1946, in Melbourne.

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METHODS.

Isolation of Virus.

The technique adopted was essentially that described by Beveridge and Burnet (1946). The patient provided a throat washing by gargling 10 ml. of saline. This was then mixed with nutrient broth and maintained at 4° C. until inoculated into the amniotic cavity of 13-day chick embryos. It was satisfactory to control bacterial contamination of the eggs by the injection of penicillin and of sodium sulphamerazine at the time of the inoculation of the throat washing. Samples of amniotic fluid were examined on the third, fourth and fifth days after inoculation, for the presence of haemagglutinin for guinea-pig or fowl cells. When this was detected, amniotic fluid and embryo lungs were harvested. An emulsion of the latter in 1 ml. of saline was used as a source of virus for further study.

Growth of Virus.

The growth of the recent strains in the egg was studied after inoculation of a volume of 0·05 ml. of material into either the amniotic cavity of 13-day embryos, or the allantoic cavity of embryos previously incubated for ten days. After inoculation the eggs were incubated at 35° C., and samples of fluid were examined at daily intervals after the first forty-eight hours. When haemagglutinin was detected, the appropriate fluid was harvested and, in the case of amniotic infection, the lungs were removed and emulsified, those from each embryo in 1 ml. of nutrient broth.

Titration of Virus Haemagglutinin.

A 2 p.c. suspension of washed cells was used.

Titration of Serum Antibody.

The technique of our agglutinin inhibition test has been described by Burnet, Beveridge, Bull and Clarke (1942). Doubling dilutions of serum were set up in volumes of 0·25 ml. in tubes of internal diameter 1 cm. and with hemispherical bottoms. An equal volume of virus containing five agglutinating doses was added and 0·25 ml. of 2 p.c. fowl red cells. The end-point was read after 45 minutes according to the pattern formed by the red cells.

INCIDENCE OF INFLUENZA A DURING 1945–46 IN VICTORIA.

During the epidemic of influenza B of October, 1945, six patients were seen who were proven to have been infected with influenza A, on four occasions by the isolation of this virus from a throat washing, and in the other cases by an examination of the serological response of the patients. In their clinical course, these six people were not distinguishable from their immediate companions who were suffering from influenza B.

After the cessation of this epidemic, no case of upper respiratory infection was seen by us until a sporadic case of type A influenza (IAN) occurred in February, 1946. The patient was a boy who had recently resumed daily attendance at school Y after the long Christmas vacation. Influenza virus was isolated from a throat washing, and a corresponding increase was noted in serum antibody.

No further case was seen until the occurrence of an epidemic became known to us on April 27th, 1946, when five boys from the same school Y reported sick. Of the 300 boys resident at this school, only 20 are known to have been affected; all the latter were seen by us during the period of one week, and proven to have been infected with influenza type A, by either seriological examination, or isolation of the virus from throat washing. Of the 900 boys attending the school daily, but returning to their own homes at night, a small number was known to be suffering from an upper respiratory infection; but none of these boys was available for our examination. Similar outbreaks of limited extent occurred among the adolescents in at least four other schools in Melbourne and Geelong during the months of May and June; representative cases from each school were proven in the laboratory to be infected with influenza A.

The clinical features of the disease were not severe. Complaint was made of initial headache and nocturnal restlessness, associated in some cases with pains in muscles and joints. Cough was present at onset in ten of these 20 cases, and developed eventually in all patients.

Epistaxis occurred in only a few cases. A temperature of 101° C. to 102° C. was recorded, but had generally returned to normal within 24 hours. Recovery was complete in three days and no sequela appeared.

The Age Incidence of the Epidemic.

The ages of children attending the schools affected ranged from seven to 17 years. The incidence of the epidemic was largely among those over eleven years, but the number of cases was not sufficient to warrant further analysis of the figures.

Trainee nurses in the Melbourne hospitals are in general less than 21 years of age. During May and June, a small number of these young women were diagnosed clinically to be suffering

from influenza. Only two were seen by us; in each case influenza type A was incriminated as the infecting agent. Sporadic cases occurred at this time among the domestic staff of the Royal Melbourne Hospital, in women whose ages were between 20 and 50 years. Two of these people were seen and a diagnosis of influenza A was established in the laboratory in both cases.

Despite specific enquiries made regarding several large groups of adults, we were unable to obtain any indication of spread of the virus through the adult community of Melbourne. Absentee figures of the staffs of several of the largest commercial establishments in the city showed no increase in May or June: the medical officer in attendance at one of these places stated that none of the 4,000 employees under her care had reported with influenzal infection. The Commonwealth Medical Officer in Melbourne, Dr. F. R. Kerr, expressed the same opinion regarding the absence of such infection at this time from among the many Commonwealth employees under his medical supervision. Routine monthly returns from the Army Medical Service showed no significant increase in the incidence of upper respiratory infection at any time during the first nine months of the year.

The conclusion seemed justified that this outbreak of influenza A selected the majority of its victims from among the younger people of Melbourne.

ISOLATION OF VIRUS.

Twenty-four attempts at isolation of virus were made from as many patients, all of whom were shown by subsequent serological tests to have been infected with influenza A. In each case the throat washing was obtained while the patient was febrile.

On eleven occasions no virus was detected during the course of two successive passages each of five days' duration in the amniotic cavity of the chick embryo. Thirteen attempts at isolation were successful; in eleven cases haemagglutinin was detected at the fourth or fifth day after inoculation of the throat washing; in the remaining two cases the haemagglutinin appeared initially during the second passage *in ovo*.

PHASE BEHAVIOUR OF THE STRAINS ISOLATED.

The strains of influenza A isolated from these cases have been studied particularly with regard to their manner of growth in the chick embryo, and the relation thereto of a change in the virus from the original "O" phase to the derived "D" phase. This variation in phase was noted originally by Burnet and Bull (1943) during their examination of strains of influenza A isolated in 1942. They observed a change in certain characteristics of the virus, occurring during successive passages at low dilution in the chick embryo. The two phases, O and D, were distinguished according to the type of fowl cell agglutination induced by the virus. Further, each virus sample was titrated against fowl and guinea-pig red cells; the ratio between these two titres (F/G) was low or zero in the case of O virus, but approximated to unity with D virus.

These workers found also, that virus from the 1942 strains was able to grow in the O phase in the amniotic but not in the allantoic cavity; but that in either situation virus in the D phase flourished readily. They suggested that O type virus gave rise to a small proportion of D type particles, and that the latter overgrew the former when the two were present in the one inoculum. However, when by suitable dilution of the O type inoculum, the small proportion of D type particles had been eliminated, this dilute inoculum initiated growth of a relatively pure O phase virus in the amniotic cavity, but could not infect in the allantoic cavity.

During the present work, the same criteria were adopted as those referred to above, in deciding the type of virus, whether O or D or an intermediate phase $\bar{o}$ or δ. The work of Burnet and Bull with the 1942 strain BEL was repeated and their findings confirmed. The results obtained were used as a measure with which the behaviour of the 1945–46 strains could be compared.

In order to clarify the description of their behaviour, these recent strains are grouped according to the dates on which they were isolated. Within each group so segregated, the characteristics of growth in the chick embryo were found to be uniform; but these characteristics varied between groups. We have regarded MIL A as typical of the A virus isolated in 1945: IAN (February, 1946) forms the second group; and CAM represents the strains isolated during the epidemic of 1946, and subsequently studied.

Growth in the Amniotic Cavity.

1. *1945 strains.* The findings with MIL A were representative of those recorded for the three other strains isolated and studied in 1945. With each strain dilutions were prepared, from 10^{-2} to 10^{-5}, of O virus in the form of first passage lung emulsion, and these dilutions were used as inocula for the amniotic cavity in batches of 13-day embryos. The phase of virus in each amniotic fluid harvested (Table 1) did not appear to be as closely correlated with the dilution of the inoculum as was the case with BEL (1942).

The O type fluid from MIL A, indicated in the Table by an asterisk, was passed in the amniotic cavity of a further batch of 13-day embryos at a dilution of 10^{-6}. The amniotic fluid harvested at three days from each of the three surviving embryos contained virus in the O phase (F/G ratio ± 0/160). Such a procedure was continued for three more passages, with similar results, the virus maintaining the O phase when passed at a dilution of 10^{-6} in the amniotic cavity.

TABLE 1.

Typical distribution of phases of virus in embryonic fluids.

A. Following amniotic inoculation of O phase virus.

Dilution of O phase inoculum.	Virus BEL.	Virus MIL A.	Virus CAM.
10^{-2}	D D ω̃ ω̃ O	δ ω̃ ω̃ ω̃ ω̃ O	D D D δ
10^{-3}	D δ δ ω̃ O	δ* ω̃ ω̃ ω̃ ω̃ O	δ ω̃ ω̃ –
10^{-4}	δ ω̃ ω̃ O – –	δ ω̃ O O* – –	D δ ω̃ O –
10^{-5}	ω̃ O O – – –	ω̃ – – – –	ω̃ ω̃ ω̃ ω̃ – –

B. Following allantoic inoculation of O phase virus.

Dilution of O phase inoculum.	Virus BEL.	Virus MIL A.	Virus IAN.	Virus CAM.
10^{-2}	D D D– – –	D δ O – –	ω̃ ω̃ ω̃ – – –	D D – – –
10^{-3}	D – – – –	δ ω̃ ω̃ O – –	ω̃ ω̃ O O –	– – – –
10^{-4}	– – – –	δ δ ω̃ ω̃ – –	O O O – –	– – – – –
10^{-5}	– – – –	D ω̃ – – – –		– – – –

A parallel series of passages, each at a dilution of 10^{-1} was commenced with the MIL A fluid in the δ phase, indicated in the Table by an asterisk. Two amniotic fluids were harvested in the second passage, they contained virus in phases ω̃ and δ. The latter at a dilution of 10^{-1} was used as inoculum for the third passage, which yielded three fluids (D, D and δ). At the fourth passage, six fluids were obtained in the D phase and of high haemagglutinin titre (F/G ± 320/400).

Thus, although it was possible with MIL A, as with BEL (1942), either to maintain the O phase or to convert to the D phase, the rate of conversion from O to D was slower in the case of MIL A and the three other 1945 strains.

2. *IAN, February, 1946.* O phase virus obtained as described later in the form of first passage amniotic fluid, was diluted 10^{-1} and inoculated into the amniotic cavity of a batch of 13-day embryos. In this way a series of five passages was commenced, each at a low dilution. The results (Table 2) are comparable with those described for MIL A; conversion from O to D phase was not complete until the fourth passage; but once established, the D phase was stable on passage in either the allantoic or the amniotic cavity.

Inoculated at high dilution, O phase virus showed no tendency to change to the D form; the O characteristics were readily retained through three passages at dilutions from 10^{-4} to 10^{-6} (Table 2).

TABLE 2.

To show development of D and O phase passages.

Dilution of inoculum.	1st passage.	2nd passage.	3rd passage.	4th passage.	5th passage.
10^{-1}	δ ω̃*	ω̃*	...	...	...
10^{-2}	...	...	δ δ* δ	D*	D D
F/G ratio of fluid harvested	25 35 / 80 140	15 / 280	80 120 50 / 160 240 160	600 / 1,000	400 350 / 400 250
10^{-4}	...	...	O O O		
10^{-5}	...	O O*	...		
10^{-6}	O* – – – –	– – – – –	...		
F/G ratio of fluid harvested	0 / 320	0 0 / 70 80	0 0 0 / 150 60 200		

The fluid indicated with an asterisk was used in each case to initiate the subsequent passage.

3. *1946 "epidemic" strains.* CAM virus in the O phase was obtained as lung emulsion following primary isolation in the chick embryo. The preparation, diluted 10–2, was inoculated into the amniotic cavity of 13-day embryos. Three of the four fluids harvested after three days contained virus in the D phase (Table 1). With higher dilutions of inoculum (10–3-10–5) the fluids harvested were of δ and ω̃ types with one O fluid at 10–4. With low dilution of inoculum, nearly complete conversion from O to D occurred during one passage, and full conversion at the second passage; in this respect CAM resembled BEL rather than MIL A. The titres of the D fluids were low, the F/G ratio of the four corresponding to the dilution 10–2 in the Table being 80/80 (D), 64/40 (D), 24/10 (D), and 32/20 (δ); subsequent repeated passage improved the haemagglutinin titre only moderately, a typical F/G ratio for a D fluid at sixth amniotic passage being 100/120.

A further six 1946 strains were examined in this manner. There was a close correspondence with CAM in four cases: the other two strains (HAY and MEY) showed a general correlation with CAM, but full D phase virus did not predominate until the third amniotic passage at low dilution.

It is felt that the rapidity of conversion from O to D phase virus in the amniotic cavity is a point of distinction between the 1942–46 epidemic strains and the 1945–46 inter-epidemic strains.

Growth in the Allantoic Cavity.

1. *1945 strains.* The findings for three A strains isolated in October, 1945, are typified by the following description of MIL A. O virus in the form of lung emulsion was prepared in dilutions ranging from 10–2 to 10–5. Embryos in batches of eight were inoculated into the allantoic cavity with each dilution. The phase of virus in each fluid harvested was found to be unrelated to the dilution of the original inoculum: examples were seen, in the fluids harvested from each batch of embryos, of several or all of the phases described by Burnet and Stone (1945) as O, ω̃, δ and D, although in the majority of the batches, virus of ω̃ and δ types occurred most frequently (Table 1 B).

The haemagglutinin titres of these fluids were uniformly low, typical figures for the F/G ratio being 6/20 for ω̃ type virus and 32/40 for D virus. Allantoic inoculation of such low titre material was repeated to the third and fourth passage, the inoculum in each case being either O or D phase virus from the previous passage. The fluids harvested from each batch of embryos, were similar to those obtained in the first passage, being of low titre and in a variety of phases, which bore no relation to the phase or dilution of the inoculum used. The simplest explanation of these findings is that virus in both the O and D phase was endowed with similar limited ability to grow in the allantoic cavity.

By contrast, D type virus which had been obtained by serial passage at low dilution in the amniotic cavity, had acquired the ability to grow well in the allantoic cavity; so that even when such amniotic fluid virus was inoculated at a dilution of 10–5, the allantoic fluid virus harvested was in the D phase and of high haemagglutinin titre (1:320). Essentially similar findings were obtained with the two other A strains isolated in 1945 and studied during growth in the allantoic cavity.

2. *Sporadic strain IAN, February, 1946.* This strain is the only A strain of all those that have been isolated in this laboratory since 1942 which could be caused to grow regularly in the allantoic cavity while retaining typical O haemagglutinating capacity.

The primary washing was obtained within a few hours of the onset of fever and positive amniotic infections were obtained with 10–1 and 10–2 dilutions of the washing as well as with undiluted material. All positive fluids had typical O characteristics.

TABLE 3.

Allantoic passage of strain IAN.

Dilution of inoculum.	1st passage.	2nd passage.	3rd passage.	4th passage.
10–1	ω̃ O O O O	...	...	...
10–2	ω̃ ω̃ ω̃ – –	O* – – –	O O O O –	...
10–3	ω̃ ω̃ O O –	O – – –	...	O O O O O
10–4	O O O* – –	O – – –	O* O O O	...
10–5	...	O – – –	...	O* O* O* O* –
10–6	...	O – – –	...	...
F/G ratio	0/28	0/48	0/70	2/160 pool.

Passage was made from the fluid indicated by an asterisk, the F/G titre in each case being shown at the foot of the column.

When one of the first passage amniotic fluids was titrated in the allantoic cavity from 10^{-1} to 10^{-4} infection occurred in most embryos and all fluids were either in the O phase or showed only weak $\bar{\omega}$ type agglutination of fowl cells. The results of further passage are shown in Table 3.

One point of interest is the extraordinary finding on second passage that in only one of four embryos in each dilution from 10^{-1} to 10^{-6} was infection evident at three days. This must indicate a wide range of susceptibility of the embryos to infection with the virus in this (temporary) phase. The results in the two subsequent passages seem to indicate that some degree of adaptation to allantoic growth had occurred, since infection was regular at dilutions of 10^{-4} or 10^{-5} and the titre of the fluids was considerably higher.

As indicated above, the behaviour of the strain in the amniotic cavity was essentially similar to that of 1942 or 1946 strains but the dominance of the full D form was only completely established after four amniotic passages at low dilution. Transfer of fifth amniotic passage D virus to the allantoic cavity gave typical high titre D fluids.

3. *Epidemic strains of 1946 illustrated by CAM.* It has not been possible to obtain from the strains isolated during the 1946 epidemic allantoic fluid containing virus in any but the D phase. O virus in the form of lung emulsion from embryos inoculated by the amniotic route, when introduced into the allantoic cavity, gave rise to infection only if the inoculum was of a low dilution such as 10^{-1} or 10^{-2}. The fluid harvested contained virus of low haemagglutinin titre, usually between 1:20 and 1:80, and in the D phase. Similar low titres were obtained in allantoic fluids with the seven 1946 epidemic strains examined, whatever type of inoculum was used. Even high titre D virus, previously prepared in the amniotic cavity, induced the growth of D phase virus of only moderate titre (1:200) when inoculated into the allantoic cavity.

In summary, it appeared that the characteristics of growth in the allantoic cavity of the epidemic strains of 1942 and of 1946 were comparable, although the latter were unable to produce high titre virus. The ability to reproduce in the O phase in this situation was an apparent distinction between the inter-epidemic strains isolated in 1945 and February 1946, on the one hand, and the 1942 and 1946 epidemic strains on the other.

Adaptation of Virus to Mouse Passage.

D phase virus in allantoic fluid obtained from fifth egg passage, was used to commence four series of mouse passages, transfer being by the intranasal route. The results obtained with the four viruses AMO (October, 1945, strain) IAN, CAM, and MEY (May, 1946, strain) did not differ significantly. The following is a description of the findings with CAM.

Two of the eight mice in the initial passage died at the fourth day with complete pulmonary consolidation. The remainder were sacrificed at five days and showed extensive pulmonary lesions; but this finding was not repeated in subsequent early passages. Second and third passage mice showed no macroscopic lesion but lung emulsion obtained from them infected the allantoic cavity of 11-day embryos at a dilution of 10^{-2}.

Lungs from two of seven fourth-passage mice killed at five days showed minimal, typical influenzal lesions; in subsequent passages areas of consolidation were found in all mice inoculated, and were considerably larger, frequently being associated with death on the fifth day. Virus was obtained by egg inoculation from such lungs and determined to be antigenically identical with CAM.

A fifth series of mouse passages was commenced with IAN O virus in allantoic fluid. No lesion was produced at any stage. Virus was recovered from first and second-passage mice but not from third-passage lungs.

DISCUSSION.

The behaviour of influenza virus infections in Melbourne during 1945 and 1946 has by no means conformed to the accepted pattern. There was an extensive epidemic of influenza A affecting Service personnel in May, 1942, and during the winter of 1943 respiratory infection in troops was associated with only a small proportion of both A and B infections. No influenza was recognized during the winters of 1944 and 1945, but a mild epidemic of influenza B developed in October–November, 1945. As has been described previously, this epidemic was practically confined to young people. It was associated with a small number of cases of influenza A which form the first group of the present study. The subsequent detection of a single case in February, 1946, made it seem highly probable that a major epidemic would develop in the following winter. Four years would then have elapsed since the preceding outbreak in 1942 and this seems to be the commonest interval between major influenza A epidemics (Stuart-Harris *et al.* 1940: The Commission on Acute Respiratory Diseases, 1946).

Virus was present in the community and if mass immunity had waned to the expected degree a winter epidemic should have resulted. Actually the epidemic which appeared was almost identical in character with the B epidemic six months previously, hardly affecting the adult population at all.

Burnet and Bull (1943) in their first description of the O–D change, suggested that influenza A virus was not capable of epidemic human spread in any but the O phase. All subsequent experience in this laboratory has confirmed the finding that influenza virus in primary isolations from human cases of the natural disease is always in the O phase. No full confirmation of the fact has yet appeared from other laboratories, but in a private communication Dr. C. H. Andrewes mentions that from influenza A cases occurring in England in the 1946–47 winter, virus has been isolated in the O phase.

If the O–D phase change is intimately related to the virulence and transmissibility of the virus, it is obviously of interest to compare in this respect strains isolated in inter-epidemic periods with those isolated from typical epidemics. As far as our limited experience has gone, one may summarize as follows:

Strains from the severe epidemic of 1942 and from the few cases in 1943 were isolated in the O phase, but readily produced D mutants which multiplied vigorously both in the amniotic cavity where they overgrew the O form, and in the allantoic cavity.

Inter-epidemic strains of spring and summer, 1945–46, were isolated in the O phase and produced D and intermediate phase variants, but these had less tendency to overgrow the O phase in the amniotic cavity. The ability of this group to produce O type virus in the allantoic cavity is the most obvious difference from the epidemic strains of 1942. It has been shown (Burnet and Stone, 1945) that a typical example of the latter group (BEL) can multiply at low titre in the O phase in the allantoic cavity but the results suggested that if the O virus multiplied sufficiently to reach levels detectable by direct haemagglutination D mutants appeared and soon overgrew the O phase. The inter-epidemic strains of 1945–46 appear to differ in three respects—(1) there is a real ability to initiate O infection in the allantoic cavity at higher dilutions; (2) there is no consistent superiority in the rate of growth, especially in the allantoic cavity, of D or intermediate mutants arising from the O phase; and (3) mutation to the full D phase occurs less rapidly. Points 2 and 3 and interdependent. As has been pointed out in earlier discussions, it is only when a well-defined mutant type has a large selective advantage over the parent type that one can obtain a clear picture of what is happening in experiments of the present type. With strain IAN there is in addition, evidence (in Table 3) that a minor mutant of the O phase capable of ready growth in the allantoic cavity was produced and selectively favoured during passage.

The strains from the 1946 minor epidemic were intermediate in character. Like those from 1942 only the D phase produced significant multiplication in the allantoic cavity. The most evident difference was the lack of vigour of growth of the D phase in the allantoic cavity.

As a working hypothesis compatible with the available facts and capable of acting as a lead for further laboratory study of the epidemiology of influenza, it may be suggested that the epidemic intensity of influenza A virus is related to the mutability of the system which is responsible for the O–D phase changes seen in the laboratory. The epidemic strains on this hypothesis are those which in the laboratory can readily mutate to D forms growing vigorously in the allantoic cavity. It is not unreasonable that increased lability of "genetic" character should allow increased opportunities for effective spread.

SUMMARY.

A mild epidemic of influenza A occurred among the adolescents of Melbourne during the winter of 1946. Rapidity of change from O to D phase, and failure to grow in the O phase in the allantoic cavity, were points of distinction between the epidemic strains, and five strains isolated from sporadic cases during the 1945–46 summer.

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SPORADIC AND MINOR EPIDEMIC INCIDENCE OF INFLUENZA A IN VICTORIA, 1945–46

II. THE BREADTH OF ANTIBODY RESPONSE FOLLOWING INFLUENZA A INFECTION

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The presence in adult sera of antibody capable of inhibiting the action of swine influenza virus has been noted frequently. The original suggestion of Laidlaw (1935) and of Shope (1936) was that the presence of this antibody indicated infection by swine virus during the 1918 pandemic. This was disproved by Stuart-Harris, Andrewes, and Smith (1938) during their study of the epidemic of influenza A in 1936 on the island of St. Helena. They found that whereas in 1935 many of the inhabitants did not possess antibody against any strain of influenza A, after the 1936 epidemic some of these same individuals exhibited antibody to both human and swine strains. Burnet and Lush (1938) suggested that the development of antibody to swine influenza was an expression of the breadth of response of the organism to an antigenic stimulus by another strain of influenza A, and that this broad type of response was found more frequently in adolescents and adults than in children.

An opportunity was afforded by the recent occurrence of a minor epidemic of influenza A in Melbourne, to study the breadth of the immunological response of a number of adolescents to a strain that was not antigenically identical with Melbourne.

TABLE 1.

Breadth of immunological response to influenza A.

Serum.	Age of patient (years).	Anti-haemagglutinin antibody to virus. CAM.	Melbourne.	WS.	SW.
SM I	7	<5	<5	<5	<10
II		20	<5	<5	<10
TOW I	16	<10	<10	<10	<10
II		320	<10	<10	<10
CAM I	16	<10	<10	10	10
II		80	160	10	10
McL I	16	20	10	10	<10
II		80	80	10	<10
MEY I	15	10	40	<10	<10
II		80	160	40	<10
WAL I	15	20	20	20	<10
II		80	160	80	<10
MEH I	19	<10	<5	<5	20
II		80	120	45	80
IAN* I	14	20	50	<10	<10
II		640	240	120	120

* Serum from the sporadic case in February, 1946.

[1]This work was carried out under a grant from the National Health and Medical Research Council.

IMMUNOLOGICAL RESPONSE BY PATIENTS.

Serum was obtained during the acute stage and a fortnight later from twenty-seven patients subsequently shown to have been suffering from influenza A in Melbourne during 1946. A well-marked gradation in the breadth of antibody response was found among these sera. Four groups of virus strains were employed in the agglutinin inhibition tests with each serum; according to the breadth of the immunological response, the twenty-seven serum pairs have been divided into four groups. The results of titration of two typical serum pairs from each group are given in Table 1. The results with virus CKS were essentially similar to those with Melbourne.

It was found that whereas sera from each of the twenty-seven patients with clinical influenza, confirmed in the laboratory, showed a rise in antibody of three-fold or more against the most recently isolated strains typified by CAM, in ten of these cases the rise in titre was confined specifically to this type of antibody. Five sera showed a rise against only CAM, Melbourne and CKS. In nine other cases, the breadth of the antibody response embraced these three viruses and WSE. Only three sera developed a significant titre of antibody to all five viruses tested, CAM, Melbourne, CKS, WSE and Swine influenza virus.

The two youngest children, aged seven and nine years, developed antibody specific for CAM. The three who produced antibody to the five viruses, were aged 14, 19, and 30 years. However, the average age of the patients did not vary significantly between the four groups in this limited series (Table 2).

TABLE 2.

Serological response to infection by 1946 A strains.

Virus to which antibody was developed.	Ages in years of patients who developed antibody following infection by CAM type virus.
CAM	7 9 11 13 14 15 15 16 16 16
CAM MEL CKS	13 14 16 16 35
CAM MEL CKS WS	11 12 13 14 15 15 15 15 22
CAM MEL CKS WS SW	14 19 30

A third bleeding was obtained from three adolescents, nine months after their infection, three individuals who previously had exhibited an antibody rise specific for CAM. This third bleeding contained antibody still strictly specific for CAM, and of approximately one-third the titre of that in the second bleeding.

Antigenic Relations between Virus Strains.

By the use of representative sera from each of these four groups, it was possible to define certain antigenic relations of recent and classical A strains (Table 3). The specific serum TOW showed a similar degree of antibody rise when tested with each of eight 1946 epidemic strains, including CAM, with four 1945 inter-epidemic strains of which MIL A is representative, and with strain IAN, derived from the sporadic case in February, 1946. A distinction is evident between the 1945–46 strains on the one hand and Melbourne, GAT, and the 1942 (BEL) and 1943 (CKS) strains on the other. No antigenic difference was detected among the 13 recent (1945-46) strains, nor between Melbourne, CKS, and BEL.

TABLE 3.

Antigenic distinctions between strains of influenza A.

Virus.	Human serum. TOW. I.	TOW. II.	McL. I.	McL. II.	MEY. I.	MEY. II.	MEH. I.	MEH. II.
1946 strains								
CAM	<10–	320	20–	80	10–	80	<10–	80
IAN	40–	320	30–	320	20–	240	10–	120
1945 strains								
MIL A	180–	800	160–	800	100–	480	40–	240
1942 strains								
BEL	<10–	<10	12–	60	<10–	60	<5–	30
CKS	10–	10	30–	160	45–	120	20–	80
Earlier strains								
Melbourne	<10–	<10	10–	80	40–	160	<5–	120
GAT	<10–	<10	<10–	25	10–	120	<5–	60
WS	<10–	<10	10–	10	<10–	40	<5–	45
SW	<10–	<10	<10–	<10	<10–	<10	20–	80

Nonspecific Inhibition of Agglutination.

Virus haemagglutinin is inhibited by high concentrations of human and animal sera; such inhibition may be nonspecific. Anderson, Burnet and Stone (1946) have emphasized that the titre of this nonspecific inhibition varies according to the sample of cells used. They used successively the cells from each of twenty-three fowls in the determination of the nonspecific inhibitory titre of a negative human serum against one virus preparation. According to the values so obtained, the samples of cells were arranged in a series, the values of the titres of one serum against one virus preparation varying from <5 to 80 through this series. These workers indicated also that the value of the nonspecific inhibitory titre of one serum, when estimated with one sample of fowl cells, varied considerably according to the strain of virus producing the haemagglutination.

Subsequent work has extended the scope of these findings. When one virus preparation was considered, such as MIL B, the order of the arrangement of the samples of fowl cells used was the same whether the negative serum was derived from human, rabbit or ferret.

The finding of a range of values for the nonspecific inhibitory titre of a serum with several samples of fowl cells, was obtained also with CAM and other recently isolated A strains. However, the range was not so extensive in these cases, nor was the order of the arrangement of the fowl cell samples the same with the recent A strains as with MIL B.

These results provide evidence of the individual nature of each virus-red cell relationship. In this respect they amplify the suggestion of a previous paper (1946) that the variation in the nonspecific inhibitory power of a serum according to the red cells used, depends in part at least on a variation in the affinity of virus for red cell.

TABLE 4.

Nonspecific inhibitory titre of negative human serum against agglutination of fowl cells by influenza virus A.

Virus.	Titre of serum.
Melbourne	<5
WS	<5
CAM	7
TAI	15
HAY	20
IAN	40
TRU	60
MIL A	180

Virus was used in the D phase. CAM, HAY and TRU are 1946 epidemic strains. MIL A and TAI were isolated in October, 1945, IAN in February, 1946.

In a study of the recent A strains, a human serum was chosen which, *in ovo*, did not inhibit infection by these viruses. Using fowl cells from one bird a determination was made of the nonspecific inhibitory titre of this serum against agglutination by WS, Melbourne, and several of the strains isolated in 1945 and 1946. Most of the recent strains were inhibited by such a negative human serum to a titre considerably higher than were the classical A strains Melbourne and WS (Table 4).

DISCUSSION.

The variable breadth of the antibody response in infection with an influenza A strain has interested workers since the first description of the neutralization of Swine virus by the sera of adults, but not of children (Laidlaw, 1935). Burnet and Lush studied the question extensively in 1938 and recently Magill and his collaborators (1944) have reopened the question of the significance of antigenic subtypes within A strains in regard to the choice of an immunizing antigen.

The information obtained in the present study is more or less confirmatory of previous work but extends it to cover a wider range of strains. Four different A types are evident, these being the infecting type CAM, and successively remote from this, Melbourne and the 1942 strains, WS and Swine. Since the practical importance of influenza immunity is concerned only with human subjects, we have felt like most other workers that serological work should be done primarily with human sera from individuals from whom the infecting strain was available.

In a previous study (Burnet, Stone and Kerr, 1946) it was shown that restricted and wide range sera could be obtained from individuals infected with influenza B, LEE B being used to detect "broad" sera.

In both studies the sera available were almost all from adolescents, so that it was impossible to confirm or refute the generalization that younger individuals provide on the whole immune sera of more restricted range than adults. Laidlaw (1935) considered that Swine type antibody was not observed under 10 years of age and it will be noted that the only two children under 10 years in our series gave completely specific antibody.

Three potentially important practical considerations arise from these results. In the first place the study of first influenza infections in young children has been largely neglected and will need to be undertaken before any clear picture of the epidemiology of influenza is available. Serological methods will be the main tool for such a study and it will clearly be necessary to have available as antigen strains correspondingly precisely to those recently current in the community studied. If haemagglutinin inhibition is used as the serological method a further difficulty is introduced by the frequent occurrence of relatively high titre non-specific inhibition of recently isolated A strains.

Secondly, if as seems reasonable, the breadth of antibody response is correlated with the breadth of resistance to clinical infection, we have at once a reasonable partial explanation of the limitation of certain epidemics, such as the two that we have recently experienced, to the younger age groups. If we extend the conception of mutability of epidemic influenza A strains developed above to include antigenic as well as O–D characteristics, it may be that a widespread epidemic affecting adults, such as that of 1937 in England, is only possible with a highly mutable strain. Only in this way can the broad antigenic resistance of most adults be overcome. The known antigenic diversity of the 1937 English strains is in accord with this view.

Thirdly, any further studies in the antigenic response to influenza vaccines may need to take into account the variable breadth of response in different individuals. Should it, for instance, become evident that a high proportion of bronchopneumonic deaths in infancy are primarily due to influenza virus infection, any attempt at immunizing this age group will probably require very close attention to the antigenic structure of the strain or strains to be used for vaccine production.

SUMMARY.

Four degrees of breadth of immunological response were noted among adolescents and young adults during the recent mild influenza A epidemic.

The use of such human sera has allowed the recognition of antigenic differences between groups of virus strains.

Nonspecific inhibition of agglutination was more marked in the case of recently isolated strains than with well adapted, older strains.

Acknowledgment. I am much indebted to Dr. F. M. Burnet for guidance in this work.

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THE DISTRIBUTION OF "T" AGGLUTININS IN HUMAN SERA

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In association with work on the changes induced in red cells by the action of mumps and influenza viruses a good deal of use has been made of the fact that such treated cells are agglutinated by practically all normal human sera. It has been found that the changes in the cells are identical with those produced by certain bacterial products and correspond to those described by Thomsen (1926) and Friedenrich (1928) as rendering cells "panagglutinable." The component of normal serum responsible for the agglutination is usually referred to as "T" agglutinin.

In a recent paper Burnet and Anderson (1947) have shown that a specific agglutinin for cells altered in this fashion can be produced by appropriate immunization of rabbits. The question therefore arose as to the possibility that "T" agglutinins in human sera were a result of auto-immunization by red cells damaged by virus, bacterial or other agents. As a preliminary approach to this problem a large series of human sera of all ages was titrated. A further series from patients with possible atypical pneumonia was also examined to compare the titre of cold agglutinins for normal human cells with the "T" agglutinin titre. As far as they go the results obtained suggest that "T" agglutinins are not immune antibodies.

TECHNIQUE.

Preparation of RD-H cells. All cells were prepared by treatment with cholera vibrio filtrates prepared as described by Burnet and Stone (1947). Details of treatment varied slightly from one batch to another, depending on whether eluted enzyme was being prepared simultaneously or not, but all the cells used were tested with our standard series of viruses and shown to be insusceptible to agglutination by ali. A standard serum Mc. with an average "T" agglutinin titre of 680 was included in all series and if the value given by this serum was outside the range 500–1,000, the other titres obtained were adjusted accordingly. Only on two occasions when a titre of 400 was obtained was this necessary. Titrations were made using doubling dilutions of serum plus an equal volume of 1 p.c. red cells, either treated or, for cold agglutinins, untreated group O human cells. The cell serum mixtures were held at 4° C. for 1½ hours after which the endpoint was read.

EXPERIMENTAL RESULTS.

"T" Agglutinins in Normal Human Sera.

In investigating the "T" agglutinin levels of normal sera, sera from maternal and the corresponding cord bloods, infants (0–6 months), infants (½–2 years), children (3–13 years) and adults (14 years and over) were examined. The results are summarized in Table 1.

Cord sera were found to contain no "T" agglutinin irrespective of the titre of the corresponding maternal serum, and very few infants less than six months of age had any "T" agglutinins in their sera. "T" agglutinins do not, therefore, pass through the placenta as do all immune antibodies.

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[1] Working under a grant from the National Health and Medical Research Council.

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TABLE 1.

"T" agglutinin titres of normal sera.

	No. in group.	Geom. mean of "T" titres.	Standard deviation of Geom. mean.
Sera from adults (14 years and over)	26	83	16
(Maternal sera)	(10)	(118)	(34)
Cord sera	12	<10	0
Sera from infants (0–6 months)	17	<10	2
Sera from infants (½–2 years)	27	24	5
Sera from children (3–13 years)	61	63	10
Differences	*p*	t	P
Adults—infants (0–6 months)	41	4·64	<0·001
Adults—infants (½–2 years)	51	3·46	0·001
Adults—children (3–13 years)	85	1·03	0·3
Children—infants (0–6 months)	76	5·63	<0·001
Children—infants (½–2 years)	86	3·56	<0·001
Infants (½–2 years)—infants (0–6 months)	42	3·15	<0·01

The mean titre of sera from infants in the 0–6 months' group was very low and differed significantly from that of infants in the ½–2 years' group and from those of the children and adult groups. The mean titre of sera from infants in the ½–2 years' group was significantly lower than those of the children and adult groups, while the difference between the mean titres of these two latter groups was not significant, indicating that children from the age of three onwards have the same mean "T" agglutinin titre as adults. In all cases a value of t corresponding to a P value of <0·01 was taken as the level of significance.

"T" Agglutinins in Sera from Cases of Primary Atypical Pneumonia.

In examining a serum from a case of primary atypical pneumonia, it was found that in addition to having a high cold agglutinin titre the serum also had a high "T" agglutinin titre. It was thought that comparison of these two titres in a number of sera from such cases might yield interesting results. A collection of atypical pneumonia sera was obtained through the kindness of Dr. P. W. Verco.

TABLE 2.

"T" agglutinin titres of sera from cases of primary atypical pneumonia.

	No. in group.	Geom. mean of "T" titres.	Standard deviation of Geom. mean.
Cold agglutinins positive	15	850	208
Cold agglutinins negative	9	90	27
Differences	*p*	t	P
Cold agglutinins positive —cold agglutinins negative	22	3·626	<0·01

Owing to the considerable variation in cold agglutinin titres obtained, using human cells of different ages and cells from different donors (Finland *et al.*, 1945), sera in this series were considered to show a significant titre of cold agglutinins if the highest titre obtained in two titrations with one to three day-old cells from different donors equalled or exceeded 30. All sera which showed a significant cold agglutinin titre were found to have a "T" agglutinin titre well above the normal level, while those which contained no cold agglutinins or whose cold agglutinin titre was less than 30 had "T" titres well within the normal range. The results are summarized in Table 2. The difference between the "T" titres in these two groups is significant.

In eight cases it was possible to obtain sera from such cases 4–6 months after convalescence. In four cases the "T" agglutinin titre remained unchanged. In the other four it had fallen to a level within the normal range. In seven cases the cold agglutinin titre had fallen to below 30. In the eighth case, one in which the "T" agglutinin level remain constant, there was an insignificant rise in the cold agglutinin titre (40–80).

"T" Agglutinins in Sera from Cases of Mumps, Influenza and Hepatitis.

As an extension of the work on atypical pneumonia, and to investigate further the possibility that "T" agglutinins were produced by auto-immunization with red cells damaged by virus, bacterial and other agents, a small number of acute and convalescent sera from cases of mumps and influenza were titrated to see if any alteration in "T" titre had occurred between the two bleedings. In the three pairs of influenza sera examined there was a rise in titre from 80–160 between the first and second sera from one case while the other two showed a fall, one from 40–30, the other from 70–15. In the two pairs of mumps sera, no alteration in "T" titre between the first and second sera could be detected. In another series, sera from a number of cases of hepatitis and other proved or suspected liver disease were titrated. The titres, however, were well within the normal range, and the figures from these patients (first bleeding only) have been included amongst the normal adults in Table 1 and Fig. 1. Acute and convalescent sera from two cases of psittacosis showed normal "T" agglutinin titres.

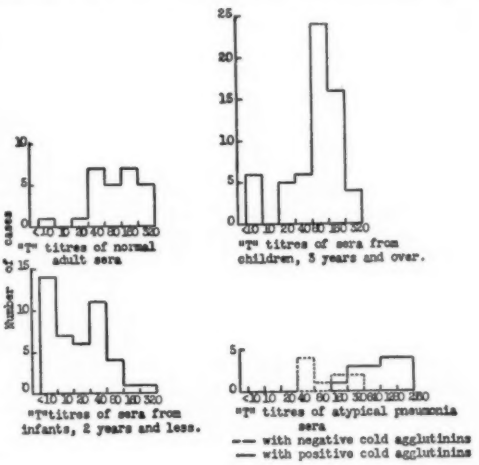
Number of cases
"T" titres of normal adult sera
"T" titres of sera from children, 5 years and over.
"T" titres of sera from infants, 2 years and less.
"T" titres of atypical pneumonia sera
— with negative cold agglutinins
— with positive cold agglutinins

Fig. 1.

Cold Agglutinins and "T" Agglutinins are Distinct.

In order to determine whether or not "T" agglutination and cold agglutination were due to the same serum component the following experiment was carried out. One in ten dilutions of an atypical pneumonia serum BRE with high cold agglutinin and "T" agglutinin titres were adsorbed with 10 p.c. packed RD-H cells for 1 hour at 0° C. and with 10 p.c. packed normal cells for three hours at 0° C. At the end of this time, the cells were spun down and washed with saline, the whole process being carried out at 0° C. The cells were then suspended to original volume in saline and held at 37° C. for one hour to allow the adsorbed agglutinins to elute. The adsorbed serum samples, the eluates from the cells used to adsorb them and the unadsorbed serum were then titrated in parallel against both 1 p.c. RD-H cells and 1 p.c. normal cells. The results are shown in Table 3 and indicate clearly that the two agglutinins are distinct.

TABLE 3.

The effect of adsorption of an atypical pneumonia serum with RD-H and normal cells.

	"T" titre.	Cold agglutinin titre.
Unadsorbed serum	3,000	220
Serum adsorbed with RD-H cells	120	160
Serum adsorbed with normal cells	3,000	30
Eluate from RD-H cells	2,000	80
Eluate from normal cells	450	160

DISCUSSION.

There is nothing in this investigation to suggest that the "T" agglutinin is in any sense an immune antibody. The failure to pass the placenta differentiates it from standard immune antibodies and the only infectious condition in which we have found an increase in titre is atypical pneumonia. The fact that the agent present in these cases is differentiable by adsorption from cold agglutinin suggests that both reactions are "accidental" manifestations of some abnormal reactive globulin of unknown significance.

SUMMARY.

A survey has been made of the titres of "T" agglutinins in human sera from individuals of various ages.

There is no "T" agglutinin in umbilical cord serum, the agent develops normally during the first year of life and after the age of three the titres are not significantly different from those of adults.

Sera from cases of atypical pneumonia with cold agglutinins are significantly higher in "T" agglutinins than normal sera. "T" agglutinins and cold agglutinins in these sera are distinct.

There is no significant alteration in the "T" agglutinin titre of sera from cases of mumps, influenza, psittacosis and hepatitis.

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THE APPLICATION OF A RECENT MODIFICATION OF THE FLUOROMETRIC METHOD OF DETERMINING RIBOFLAVIN TO SOME SAMPLES OF AUSTRALIAN WHEAT AND WHEAT PRODUCTS

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A considerable amount of research has already been done in an endeavour to find a satisfactory method of determining the riboflavin content of cereals. In collaborative assays microbiological methods have shown considerable differences between the results obtained on the same sample of wheat by workers in different laboratories (Andrews, 1943). Biological assays with rats have given results for wheat and wheat products of a much higher order than most microbiological or fluorometric methods (Copping, 1943, 1946). When applied to the determination of riboflavin in urine, widely-used fluorometric methods have revealed a lack of specificity which might also be expected in the analysis of cereals with these methods (Morell and Slater, 1946).

A modification of the fluorometric method, highly specific for the determination of riboflavin, has been developed by Slater and Morell (1946). The present paper describes the application of this method to the analysis of riboflavin in some Australian wheats and wheat fractions.

EXPERIMENTAL.

Sampling.

Samples of wheat, flour, bran, pollard and germ were obtained from one mill in each State. The wheat fractions were drawn from the mill streams shortly after the wheat was collected, to ensure, as far as possible, that the mill products were from wheat similar to that sampled. All samples were ground very finely in a household cereal grinder, thoroughly mixed and stored in the dark until analysed.

Method of Analysis.

In the analysis of bran, a slight modification (to be described in detail later) was applied in the extraction procedure. Otherwise the analysis of all products was carried out in the following manner:

The weighed sample (wheat, pollard and bran, 10 gm.; flour, 20 gm.; germ, 8 gm.) was briefly shaken with 150 ml. 0·1 N. HCl and autoclaved for 15 minutes at 15 lb. pressure. After cooling, 1 ml. 3·2 p.c. KCN was added to activate the papain and the pH adjusted to 4·5. 0·4 gm. takadiastase (Parke-Davis, Sydney) dispersed in 20 ml. water and 0·4 gm. papain (Parke-Davis, Sydney) mixed with an equal amount of glycerin and dispersed in 20 ml. water, were added, the mixture shaken and incubated overnight at 37° C. The mixture was then filtered (No. 1 Whatman filter paper), the residue washed several times with hot water and the filtrate made up to 200 ml.

Aliquots (wheat, pollard and bran, 5 ml.; flour 10 ml.; germ, 3 ml.) were taken for measurement of the riboflavin content by the method of Slater and Morell (1946). 3 ml. 6 p.c. $KMnO_4$ (freshly prepared) were used for the oxidation of impurities in all aliquots and 7 gm. Na_2SO_4 employed for the saturation of the aqueous layer in the case of flour extraction aliquots; 3 gm. Na_2SO_4 were used for all other aliquots. Frothing was considerably reduced during the decolourization of the permanganate by peroxide by adding six drops of butanol-pyridine solution previous to the addition of the peroxide.

Recovery analyses, which were carried out each day and which included all types of samples, were made as above with the following differences: 5 ml. 20 μg/ml. riboflavin solution were added to the sample before extraction, 2 ml. aliquots were always taken for riboflavin determination and 2 ml. 6 p.c. $KMnO_4$ used in the oxidation procedure. If recoveries were below 90 p.c., the accompanying batch of analyses was rejected.

Reproducibility of Method.

The reproducibility of this method applied to wheat was determined by analysing the same wheat on ten occasions. Results: 0·818, 0·750, 0·768, 0·714, 0·728, 0·754, 0·780, 0·753, 0·802, 0·724; mean 0·759; standard deviation, 0·0337; coefficient of variation, 4·44 p.c. The percentage error of the method (if one defines the error as a quantity X such that 95 p.c. of results will lie within mean $\mp$ X) is about $2 \times 4{\cdot}44 = 8{\cdot}9$ p.c.

Examination of Various Extraction Procedures.

In order to find the most suitable and complete extraction method, five different extraction procedures were studied.

1. 10 gm. finely-ground wheat were shaken with 150 ml. acetate buffer pH 4·5. 20 ml. aqueous suspensions of 0·2 gm. takadiastase and papain (activated by 1 ml. 3·2 p.c. KCN) were added, the mixture thoroughly shaken and then incubated overnight at 37° C. After making up to 200 ml. with distilled water, the mixture was filtered and the riboflavin content determined on 5 ml. aliquots.
2. As in 1, except that 0·2 gm. polidase-S (Schwarz Laboratories, New York) was used instead of takadiastase.
3. As in 1, except that 0·2 gm. clarase (Takamine Laboratory, New York) was used instead of takadiastase.
4. As previously described for the general analysis for wheat, except that the extraction mixture was filtered *after* making up to 200 ml.
5. 10 gm. wheat were autoclaved with 150 ml. 0·1 N HCl for fifteen minutes at 15 lb. pressure. After cooling, the volume was made up to 200 ml., the mixture filtered and the riboflavin content determined on 5 ml. aliquots.

The results shown in Table 1 were obtained after correcting for the riboflavin content of the enzymes (determined in the same way) and for the volume occupied in the extracts by undigested wheat.

TABLE 1.

Riboflavin content of wheat, using different extraction procedures.

Extraction procedure.	Riboflavin content μg/gm.	Residue as p.c. original weight.	Recovery of added riboflavin.
1	0·80	78	101
2	0·77	72	94
3	0·78	78	100
4	0·83	16	95
5	0·80	39	95

It may be assumed from these results that the most complete extraction procedure (No. 4) which left only 16 p.c. of the wheat undigested, liberated no more riboflavin than the least complete procedure (78 p.c. undigested).

Special Extraction Procedure for Bran.

Recovery analyses on bran samples invariably showed recoveries of between 80 and 90 p.c. Low recoveries may be due either to destruction of riboflavin during analysis (including extraction) or to loss of riboflavin by adsorption on to undigested material. Since the method of extraction of bran was the same as for the other wheat products, it was unlikely that the low recoveries were due to destruction of riboflavin. That the riboflavin was adsorbed on to the undigested residue was shown by the following experiment:

10 gm. bran plus 5 ml. 20 μg/gm. riboflavin solution were extracted according to the usual procedure to the filtration stage. After filtration, the residue was washed with hot water and the filtrate made up to 200 ml. (extract A). The riboflavin content was determined on 5 ml. aliquots. The residue was washed, in two 25 ml. portions with 50 ml. eluting solution (pyridine 30 vol., water 35 vol. and glacial acetic acid 2 vol.). The residue was again washed with hot water and the combined eluates and washings made up to a convenient volume (extract B). The riboflavin content of the solution was determined on 5 ml. aliquots.

An analysis of bran, without added riboflavin, was made, using the elution technique, at the same time as the recovery analysis (extract C). The experiment was done in duplicate on a separate occasion and the results are shown in Table 2.

TABLE 2.

Recovery of adsorbed riboflavin from undigested bran residue.

Extract C riboflavin µg.	Extract A riboflavin µg.	Extract B riboflavin µg.	P.c. recovery of added riboflavin.
23·3	112·6	13·2	$125\cdot8-23\cdot3-\frac{100-(112\cdot6-23\cdot3)}{100}\times 23\cdot3=100\cdot0$
24·3	119·0	7·4	$126\cdot4-24\cdot3-\frac{100-(119\cdot0-24\cdot3)}{100}\times 24\cdot3=100\cdot7$

The results in Table 2 show that significant recoveries of adsorbed riboflavin were effected by washing the residue with pyridine-acetic acid eluting solution. This modification of the extraction procedure was therefore used in all bran analyses shown in Table 4.

Specificity of Method of Analysis.

Slater and Morell (1946) have described an easy check on the specificity of the method used to determine the riboflavin content of aliquots from extracts of biological materials. Essentially, this check involves three exposures to sunlight of the butanol-pyridine extract of riboflavin, readings of the fluorescence being taken after each exposure. Non-riboflavin, light-labile, fluorescing compounds, if present, would cause a trend in the answers worked out for each exposure, the direction of the trend depending upon whether the interfering compound was decomposing faster or slower than riboflavin. Only such compounds having a decomposition rate very similar to that of riboflavin, which Slater and Morell (1946) have shown to be unusual, could be measured as riboflavin.

In Table 3 the results for each exposure for each mill product are given together with the per cent. destruction of riboflavin.

TABLE 3.

Results of specificity tests on all types of samples analysed.

Product.	Riboflavin content calculated for each exposure. µg./gm.	p.c. destruction of riboflavin. gm.
Wheat	0·77	48
	0·77	71
	0·76	92
Flour	0·51	63
	0·52	85
	0·50	93
Bran	2·5	53
	2·25	88
	2·36	93
Pollard	1·53	55
	1·55	77
	1·6	84
Germ	5·2	46
	5·0	70
	5·0	90

The results in Table 3 show that a high degree of specificity has been obtained. Where specificity tests have been done resulting in an upward or downward trend in the three results, it has been the experience of this laboratory that an increase in the amount of $KMnO_4$ used for oxidation of impurities removes the trend.

RESULTS.

The riboflavin contents of samples of wheat and its mill products obtained from six different mills are given in Table 4. These figures represent single determinations except where it is otherwise indicated.

TABLE 4.

Riboflavin content of wheat and wheat products.

Mill product.	Mill.	Riboflavin µg./gm.	Average riboflavin content of samples * µg./gm.	Products as p.c. extractions of whole wheat.	Riboflavin in products; p.c. of whole wheat.
Wheat	1	0·79		100	100
	2	0·80—		100	100
	3	0·80	0·835	100	100
	4	0·95—		100	100
	5	0·81—		100	100
	6	0·88		100	100
Flour	1	0·26		71·5	23·6
	2	0·31—		71	27·5
	3	0·26	0·271	72	23·4
	4	0·27		70	19·9
	5	0·25		72	22·2
	6	0·28		71·3	22·6
Bran	1	2·3		12·5	36·4
	2	2·26—		7·3	20·6
	3	2·3	2·35	13·2	38·0
	4	2·69		13·0	36·8
	5	2·2		16	43·5
	6	2·48		18	50·6
Pollard	1	2·23—		16·0	45·2
	2	1·61—		21·7	43·6
	3	1·76	2·07	14·8	32·5
	4	2·11		17·0	37·7
	5	2·31		11·8	32·4
	6	2·37		10·4	28·0
Germ†	1	—		—	—
	2	—		—	—
	3	5·2	4·25	0·25	1·7
	4	4·0		0·25	1·1
	5	4·8		0·25	1·5
	6	3·8		0·25	1·1

* These figures represent the means only of the samples obtained from six mills and are not necessarily average values for Australian wheat and wheat products.

† Mills 1 and 2 did not separate off the germ in their milling process.

— Duplicate determinations.

In Table 5 the total riboflavin content determined in the wheat fractions for each mill are compared, as a percentage, with the riboflavin content determined for the whole wheat sample from the same mill.

TABLE 5.

Percentage relationship between the riboflavin content of the wheat fractions and that of the whole wheat.

Mills.	Riboflavin content of wheat µg./100 gm. 1	Total contribution of riboflavin in products to 100 gm. wheat. 11	$\frac{11 \times 100}{1}$
1	79	83·1	105
2	80	73·4	91·7
3	80	75·1	93·9
4	95	90·7	95·5
5	81	81·6	100·8
6	88	90·1	102·3

The results given in Table 5 show a close agreement between the riboflavin content of the wheat fractions and the riboflavin content of the whole wheat.

DISCUSSION.

The results given in Table 4 for some Australian wheat and wheat products are somewhat lower than those published by previous workers for similarly designated samples. Since the methods used for the determination of riboflavin vary with different workers and since the samples analysed by them have been milled by varying techniques, it is difficult to compare the results obtained in different countries for wheat fractions.

Barton-Wright and Booth (1943), using the microbiological method, found the riboflavin content of some Australian wheat samples to be 2·4–2·65 μg/gm. These figures are considerably higher than those found in this survey, viz. av. 0·84 μg/gm. Copping (1946) has reported the riboflavin content of an English-milled whole wheat flour, determined both macrobiologically and microbiologically, to be 2·3 μg/gm. and 2·78 μg/gm. respectively. Kodicek (1946) has compared results of microbiological and fluorometric determinations on 100 p.c. extraction flour, with similar determinations by other workers and the average results were all within the range 1·0–1·3 μg/gm.

The lower results for the riboflavin content of the Australian white flours studied by the author (average 0·27 μg/gm.) if confirmed by a representative survey of Australian flours, are of importance from the point of view of human intake of riboflavin. Kodicek (1946) found the riboflavin content of 70 p.c. extraction flour analysed by the fluorometric and microbiological methods to be 0·36 and 0·32 μg/gm. respectively. In his survey of the results of several workers, Kodicek gave a range of 0·35–0·40 μg/gm. for "similar white flours." In a report on a collaborative assay of the riboflavin content of white flour, Kemmerer (1944) reports a value representative of collaborators' results of 0·4 μg/gm.

The values for bran, pollard and germ given in this report are also lower than the majority of those reported in the literature. Conner and Straub (1941) have published figures for germ of the same order as shown above.

Some of the discrepancy between results obtained by fluorometric and microbiological methods may be due to the questionable validity of assays using the "single curve" method of computing microbiological assay results. Wood (1945) has strongly criticized this method and has shown that where differences in the blanks of the standard of test solutions occur (as seems often the case), the "single curve" method of computation may give results considerably higher than those worked out by the "slope ratio" method. An actual assay cited by Wood showed a difference of about 30 p.c.

Microbiological assays are most reliable when the blanks for standard and test solutions are identical. Price (1945) points out that although the difference in blanks "may not be statistically significant in a *single experiment*, there is cause for concern in the fact that it is usually positive." Using an enriched media (one containing more of the non-riboflavin stimulating substances) Price (1945) claims considerably lower results for extracts which gave a positive difference between the blanks of test and standard solutions. He suggests that "for the assay of cereals with the media at present in use".... "the lower, rather than the higher results are likely to be closer estimates of the riboflavin content."

Most of the microbiological determinations published have been carried out by methods having the disadvantages mentioned above. They must therefore be suspected of giving high results.

Wood (1946) makes a further criticism of the microbiological assay procedure. He points out that "while the result of any statistically invalid[1] assay must be for that reason in error, it does not follow that the result of a statistically valid assay is not in error. If, for example, in a riboflavine assay the Test Preparation should happen to contain not only riboflavine, but also some other growth-stimulating factor, and if this other factor stimulated growth proportionally to the dosage at all dosage-levels, no statistical test and no method of calculating the result could possibly detect anything suspicious in the result obtained. The combined riboflavine and other factor would be estimated as riboflavine." Thus, it seems not only that many published microbiological assays may have been statistically invalid, but, in addition, possibly nonspecific for riboflavin.

[1]Used in a restricted statistical sense.

The lack of specificity of several fluorometric methods, shown by Morell and Slater (1946), has already been referred to and further indicates the hitherto unsatisfactory position of the riboflavin analysis of cereals. Although low results have been obtained in this laboratory for wheat, etc., it has been shown in this paper that:

(1) complete extraction or extractions at least as complete as those obtained by other workers have been used;
(2) no loss of riboflavin, as shown by overall recoveries, has occurred during the whole analysis procedure; and
(3) the method gives a high degree of reproducibility and is highly specific for riboflavin.

SUMMARY.

The method of Slater and Morell has been applied to the determination of riboflavin in wheat and wheat fractions. Average values for six wheats, flours, brans, pollards and four germs were 0·84, 0·27, 2·35, 2·07 and 4·25 μg/gm. respectively.

Methods of extracting riboflavin from wheat have been examined for suitability and completeness of extraction. A special procedure was adopted for bran to prevent loss of riboflavin by adsorption on bran residues.

The specificity of the methods used for estimating each product was checked and found to be satisfactory.

The possibility is discussed that the lower results found for some Australian wheat samples may be due to increased specificity of the method used for determining riboflavin.

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STUDIES IN INFECTIOUS ECTROMELIA OF MICE

I. IMMUNIZATION OF MICE AGAINST ECTROMELIA WITH LIVING VACCINIA VIRUS.

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The recent demonstration by Burnet and Boake (1946) of the close relationship which exists between the viruses of infectious ectromelia and vaccinia has stimulated us to study again the epidemiology of ectromelia in mice by the methods of experimental epidemiology developed by Topley and his colleagues (Greenwood, Hill, Topley and Wilson, 1936). The development of effective methods of immunizing mice against ectromelia by previous inoculation with vaccinia virus, the use of the chorioallantois of the developing egg (Burnet and Lush, 1936) and the haemagglutinin test for the detection and titration of ectromelia virus, and the haemagglutinin-inhibition test for the presence of antibody in the serum of infected animals provide methods of study not previously available.

The present paper is the first of a series of studies designed to clarify certain aspects of the immunology and epidemiology of the disease before commencing experiments involving the spontaneous spread of ectromelia in large colonies of mice. It is concerned with the elaboration of Burnet and Boake's (1946) observation that mice could be immunized against ectromelia by the intravenous inoculation of vaccinia virus.

Several workers have studied the response of mice to vaccinia virus inoculated by various routes. Their results differed greatly, apparently because of the varying virulence for mice of the different strains of vaccinia virus used. In view of these conflicting reports, the effects were also studied of the inoculation by various routes of mice of the Hall Institute stock with the "Gillard" strain of vaccinia virus.

MATERIALS AND METHODS.

Virus. Two strains of ectromelia virus have been used. The "Hampstead" strain was originally isolated by Marchal (1930). It had undergone a number of chorioallantoic passages in 1935-6, and had been stored since then in the dried state. In 1945 it was revived by chorioallantoic passage and has been maintained in that way. In June, 1946, we received from Dr. C. H. Andrewes of the National Institute of Medical Research, Hampstead, a Russian strain of virus, which we have called the "Moscow" strain, in the form of dried mouse liver. This strain was isolated from a spontaneous outbreak in Professor V. D. Soloviev's Moscow laboratory. The material used in most experiments has been passaged once in mice and then once on the chorioallantois.

The vaccinia strain "Gillard" was derived from an American commercial vaccine lymph. Other vaccinia strains used in comparative experiments were "C.S.L." (Australian standard strain) and "Java," strains of dermal vaccine, and Levaditi's "Neurovaccine." All strains had undergone several chorioallantoic passages.

Stock suspensions of virus were obtained by inoculating a series of eleven or twelve-day old chick embryos on the chorioallantois by Burnet's method (Beveridge and Burnet, 1946) and harvesting the membranes after incubation at 35° C. for two days (for vaccinia virus) or three days (for ectromelia virus). The membranes were allowed to drain in the refrigerator for a few hours and were then ground with sterile sand and taken up in

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"calcium saline" (0·85 p.c. NaCl, 0·024 p.c. $CaCl_2$), 0·5 ml. for each membrane. The supernatant fluid obtained after centrifugation for ten minutes in a small angle centrifuge was used as the stock virus suspension and was designated "undiluted" virus suspension.

The virus content of both ectromelia and vaccinia virus suspensions was determined by chorioallantoic titration, six embryos being used for each ten-fold dilution. Virus suspensions were titrated for haemagglutinin by Nagler's (1944) method, using fully susceptible fowl's cells. When suspensions which were several days' old were used for haemagglutinin-inhibition tests the preliminary titration of the haemagglutinin content was carried out with 5 p.c. normal mouse or rabbit serum in saline, to inhibit the nonspecific lipid haemagglutination found in such suspensions (Stone, 1946).

Mice. In most experiments eight-weeks-old male mice of mixed colours were used. The mice were all bred in the Hall Institute mouse-room and no intercurrent disease has been observed amongst them.

Control animals used in experiments extending over a considerable period of time or involving mice of various ages were of the same age as the vaccinated mice used in these experiments.

Inoculations were made by standard methods. The anaesthetic used for intranasal inoculation consisted of one part of chloroform and two parts of ether.

Infection with ectromelia after intraperitoneal challenge inoculations was judged by the characteristic changes in liver, spleen, and intestines, the presence of large amounts of free fluid in pleural and peritoneal cavities, and intra-abdominal fat necrosis. Partially immune mice often remained healthy after the challenge inoculation but showed minimal lesions of ectromelia at autopsy. The commonest finding in such animals was a white exudate on the spleen.

Estimation of Ectromelia or Vaccinia Antihaemagglutinin in the Serum.

Ectromelia antihaemagglutinin (E-AHA) and vaccinia antihaemagglutinin (V-AHA) were estimated as follows. Normal agglutinins for fowl red cells, which occur in many mouse sera, were absorbed with a drop of packed fowl cells. The inactivated serum was diluted to 1/5 and 0·25 ml. of this dilution was added to 0·25 ml. of 2 p.c. washed susceptible fowl cells in a round-bottomed glass tube. The serum was diluted in serial two-fold dilutions in 1 p.c. fowl cells. To each tube 0·25 ml. of a dilution of ectromelia or vaccinia virus adjusted to contain four haemagglutinating doses of virus was added. After shaking, the rack was placed in the incubator at 37° C. for 20 minutes, reshaken and finally examined after one hour at 37° C. The endpoint was a certain degree of partial agglutination taken as one plus (+). The titre of E-AHA or V-AHA is expressed as the reciprocal of the dilution of serum in red cells prior to the addition of virus.

In some experiments the titre of E-AHA or V-AHA was estimated before the vaccinated animal was challenged with ectromelia virus. The mouse was bled from the tail veins into a capillary tube, one end of which was then sealed. After centrifugation a small volume of packed fowl's cells was introduced into the tube with a drawn-out Pasteur pipette and mixed with the serum by centrifugation and shaking for half an hour, thus absorbing fowl cell agglutinins. The serum was clarified by further centrifugation and with a drawn-out Pasteur pipette graduated to the tip 0·01 or 0·02 ml. serum was removed and added to 0·04 or 0·03 ml. saline in a narrow round-bottomed tube and inactivated; 0·05 ml. p.c. susceptible fowl's cells were added to this tube, and the mixture diluted out in 0·05 ml. volumes of 1 p.c. fowl's cells. After the addition of 0·05 ml. of virus suspension containing four haemagglutinating doses, the racks were shaken and read after an hour in the incubator just as in the standard method using 0·25 ml. volumes. Estimations carried out on the same specimens of serum by the two methods showed close agreement.

In sera examined after recovery of mice from infection with vaccinia or ectromelia, there is usually a difference of about one tube in the V-AHA and E-AHA titres, i.e. the titre of the inhibition of haemagglutination by the homologous virus is about twice the titre of the inhibition of haemagglutination by the heterologous virus.

Estimation of Vaccinia Virus Content of Organs of Mice.

The course of vaccinia infections in mice was followed by titrating blood and suspensions of organs on the chorioallantois of chick embryos. At intervals after inoculation of the vaccinia, virus animals were sacrificed by being bled from the axilla under anaesthesia and the blood treated in various ways, being either defibrinated and plasma and cells tested separately for the presence of virus, or diluted in an equal volume of citrate saline and tested for virus. Serum was also obtained for antibody tests. Autopsy was carried out with instruments separately sterilized before the removal of each organ. After removal, the various organs were ground with sterile sand, taken up in 0·5 ml. of sterile normal horse serum saline, centrifuged, and the supernatant fluid diluted appropriately in calcium saline and inoculated in the chorioallantois of twelve-day old chick embryos. These were examined and foci of vaccinia counted two days later.

In certain experiments the tail, or a piece of infected skin, was removed, cut into small pieces, ground with sand in a mortar, taken up in 2 ml. saline, centrifuged and the supernatant treated for five minutes with one-third of its volume of ether before chorioallantoic inoculation.

The organs of some mice were fixed in formol-saline and sections were cut and stained with haematoxylin and eosin.

EXPERIMENTAL RESULTS.

Intravenous Inoculation of Vaccinia Virus.

Eyer (1937) using neurovaccinia virus adapted to mouse-brain, found virus in the lungs, liver, spleen, testis and kidney six days after intravenous inoculation. The animals did not appear sick, but histological examination of the organs showed changes in the lungs, liver, and spleen, chiefly congestion and focal round-cell infiltration.

The only signs of infection following the intravenous inoculation of mice with "Gillard" vaccinia were oedema, engorgement, and scabby lesions of the tail, first seen two days after the injection, reaching maximum intensity at the end of a week and then gradually disappearing. Such reactions were not seen after the injection of dilutions of virus greater than 1:10,000. The lesions on the tail were more severe when tails had been dipped in hot water prior to the intravenous inoculation.

Tests for the presence of virus in several organs at daily intervals from the first to the seventh day and on the tenth day after inoculation failed to show virus in the blood after the first 24 hours. Virus was never found in the liver and only once, on the fifth day, in the spleen. A small amount of virus was present in suspensions of the superficial lymph nodes and the lungs on the fourth, fifth and sixth days. Vaccinia virus was first isolated from the tail on the third day. It increased in amount to reach a maximum concentration on the sixth day, thereafter decreasing until it had disappeared entirely by the tenth day. (Table 5).

The antibody response to the intravenous inoculation of various dilutions of vaccinia virus was determined 13 days after the injections by bleeding the mice from the tail veins and determining the vaccinia anti-haemagglutinin titre of the sera obtained. The results are shown in Table 1, from which it can be seen that low titre V-AHA is produced irregularly after the intravenous injection of dilutions of vaccinia from 1:10,000 to 1:1,000,000; low titre V-AHA is regularly produced after dilutions of 1:1,000; and at lower dilutions V-AHA is produced regularly and usually reaches a high titre.

The groups of mice whose sera were tested for antibody content on the thirteenth day after the intravenous injection of various dilutions of vaccinia virus were inoculated intraperitoneally the next day with 0·2 ml. of 1:10 dilution of ectromelia virus, half being challenged with the "Hampstead" and half with the "Moscow" strain. The results are shown in Table 1, in which the subsequent fate of the mouse can be compared with the titre of V-AHA in its serum prior to the challenge inoculation. Some protection, shown by prolongation of life, was evident even at the highest dilution (1:1,000,000) when the "Hampstead" strain was used for the challenge, and after inoculation with concentrations of vaccinia virus greater than 1:10,000 no deaths occurred and no mice showed severe lesions when killed 14 days after the challenge inoculation.

The inoculation of the "Moscow" strain was a much more severe test and previous injection with concentrations of vaccinia of 1:100,000 and 1:1,000,000 had no effect on the mortality. Deaths due to ectromelia occurred even after immunization of mice by the intravenous inoculation of undiluted vaccinia virus. Of the ten deaths occurring in the 47 mice of groups in which some mice survived, all but three occurred after a normal incubation period of three to six days and the mice showed the typical acute specific changes seen in the controls. The three mice that lived for nine, eleven and thirteen days, respectively, all developed conjunctivitis and skin lesions, and on post-mortem showed adhesive peritonitis, fat necrosis of intra-abdominal fat and necrosis of the edge of the liver and of the spleen. The lesions were quite similar to those seen in non-immunized mice dying at a similar period after infection with the "Hampstead" strain of the virus or with high dilutions of the "Moscow" strain. Deaths usually occurred in mice which had not developed a high V-AHA titre.

Intranasal Inoculation of Vaccinia Virus.

Nelson (1938) and Lush (1940) obtained very different results following the intranasal inoculation of mice with vaccinia virus. Nelson used virus grown on the chorioallantois from a suspension of elementary bodies given him by Dr. T. M. Rivers. He found that the nasal instillation of suspensions of infected egg-membranes in dilutions up to 1:1,000 caused coryza and pneumonia, with a high mortality rate. The disease was transmissible by passage of the nasal exudate. Lush, using Lister lapine (dermal) strain of vaccinia virus and the Mill Hill mice, was unable to demonstrate multiplication of virus in the lungs. Iguchi (1939),

TABLE 1.

Immunization of mice against ectromelia with living "Gillard" vaccinia virus (chorioallantoic titre $2{\cdot}8 \times 10^8$ infective particles per ml.) 0·05 ml. inoculated intravenously.

Dilution of vaccinia virus.		Challenge with ectromelia virus 1: 10, 0·2 ml. by intraperitoneal inoculation. "Hampstead" strain ($2{\cdot}8 \times 10^8$ infective particles per ml.)						"Moscow" strain. ($3{\cdot}7 \times 10^8$ infective particles per ml.).												V-AHA titre. Geometric mean.[6] ± S.E.
10-6	V-AHA before challenge	10*		0			10	<5	<5				5							
	Result of challenge	5†	5	10	11	14	+‖	4	4	5	5	6	6							
10-5	V-AHA before challenge	7		5	<5				<5	<5	<5									
	Result of challenge	5	12	+	+	+	+	3	4	4	4	5	8							
10-4	V-AHA before challenge		<5	<5			10	5	<5		40									
	Result of challenge	5	6	+	+	±§	—¶	3	4	9	—	—	—							
10-3	V-AHA before challenge				10	10	5			7	10	80								12± 5
	Result of challenge	±	±	±	±	—	—	4	5	6	11	±	±							
10-2	V-AHA before challenge	10	640	160	160	80	40	10	40	40		640	320	80	50	40	80	80		78±24
	Result of challenge	±	—	—	—	—	—	4	13	+	+	±	±	±	±	±	—	—		
10-1	V-AHA before challenge	400	320	320	200	200	80	320	320	40	1,000	640	640	640	640	400	320	320	160	310±57
	Result of challenge	—	—	—	—	—	—	±	±	±	—	—	—	—	—	—	—	—	—	
Undiluted	V-AHA before challenge	400	640	400	400	320	320	80	640	320	1,000	640	640	400	320	320	320	320	160	370±50
	Result of challenge	±	—	—	—	—	—	4	±	±	—	—	—	—	—	—	—	—	—	
Controls		5	5	5	5	5	5	6	9	12				3	4	4	4	4	4	5	5	5

* Reciprocal of dilution of serum at which four agglutinating doses of vaccinia virus caused "+" haemagglutination.

†5 etc. = deaths from ectromelia 5 etc. days after challenge inoculation.

‖+ = survival for 14 days after challenge inoculation with severe chronic lesions of ectromelia found at post-mortem.

§± = survival for 14 days after challenge inoculation with very slight chronic lesions of ectromelia found at post-mortem.

¶— = survival for 14 days after challenge inoculation with no lesions of ectromelia at post-mortem.

[6] For simplicity, the standard error has been converted from a factorial to the equivalent additive number.

TABLE 2.

Vaccinia virus in the organs of mice killed at intervals after the intranasal inoculation of 1:100 "Gillard" vaccinia virus (chorioallantoic titre 6·0 × 10^8 infective particles per ml.)

Time after inoculation.	Number examined.	Titre of vaccinia virus*. Cervical lymph nodes undiluted.	Nasal mucosa scrapings. 10–2	10–1	Undiluted.	Lungs. 10–2	10–1	Undiluted.	V-AHA in serum. Titre†.
3 hours	2	0, 0	..	0, 0	1, 7	9, 7	32, 36	++, ..	
22 hours	2	0, 0	..	0, 0	0, 5	0, 0	4, 2	.., 13	
2 days	2	0, 0	0, 6	1, 40	9, ++	.., 5	3, 65	37, +++	0
3 days	2	0, 0	47, 65	++, ++	++, +++	15, 9	80, 50	+++, ++	0
4 days	2	0, 0	5, 5	60, 35	+++, ++	14, 8	50, 73	++, ++	0
5 days	2	3, 5	3, 5	37, ..	.., +++	5, 30	87, ++	..	0
6 days	2	0, 4	22, 11	90, ++	..	16, 9	++, ..	..	0
7 days	2	0, 0	12, 0	++, 0	..	12, 1	50, 9	..	10
10 days	2	0, 0	..	..	0, 0	..	.., 2	0, ..	320, 320, 400
14 days	4	0, 0, 0, 0	..	..	0, 0, 0, 0	..	..	0, 0, 0, 0	160, 240, 320, 320, 640

* Expressed as numbers of foci (average of counts on two membranes) produced by suspensions from individual mice.

+++ = confluent lesions, ++ semiconfluent lesions.

† Reciprocal of dilution of serum at which four agglutinating doses of vaccinia virus caused "+" haemagglutination.

working with a neurovaccinia virus adapted to mouse-brain, obtained results intermediate between these two extremes. Inapparent infections followed the intranasal instillation of the virus and produced immunity to subsequent intracerebral challenge inoculations of vaccinia virus.

Preliminary experiments with a 1:100 dilution of "Gillard" vaccinia virus were not attended with any sickness or mortality, and the behaviour of the virus after intranasal instillation was investigated in some detail. When undiluted virus was used the mice invariably became sick, with ruffled fur, loss of weight and rapid respiration. Four out of 44 mice died between the fifth and the eighth day, the major finding at post-mortem being the extensive rather patchy greyish consolidation of the lungs. Nelson found in two out of five contact experiments that normal mice could receive an immunizing dose of virus by cohabitation with infected animals. In two experiments six normal mice were placed with twelve infected mice in a tin ten inches in diameter. After twenty-one days one contact mouse showed evidence of inapparent infection by the development of V-AHA and immunity to challenge inoculation with "Hampstead" ectromelia virus. The other eleven failed to develop V-AHA and died of ectromelia within six days of the challenge inoculation.

With dilutions of 1:10, or higher, the mice remained healthy, but regularly developed V-AHA to a high titre after the inoculation of doses of dilutions up to 1:10,000, and irregularly showed low titre V-AHA after doses of 1:100,000 and 1:1,000,000. Table 3 sets out the results in detail. The titres were slightly higher than those following the intravenous inoculation of the same dilutions of virus.

The behaviour of the virus after the intranasal inoculation of 0·05 ml. of a dilution of 1:100 suspension was followed by killing mice at daily intervals and testing suspensions of different organs on the chorioallantois for their virus content. Culture of the blood, liver and spleen always gave negative results. The virus multiplied on the nasal mucosa and in the lungs between the third and the seventh day. The results are shown in Table 2. The only macroscopic change in the lungs of these mice was congestion, most pronounced between the fourth and seventh days. Microscopically there was also patchy infiltration of the inter-alveolar septa with lymphocytes and mononuclear cells. The cervical lymph nodes were enlarged between the second and sixth days after inoculation and contained virus on the fifth and sixth days. No changes were seen in the sections of nasal mucosa examined.

Histological examination of the nasal mucosa and the lungs of mice killed at intervals after the inoculation of undiluted virus showed changes similar to those described by Nelson.

The experiments on the immunity to ectromelia conferred by the intranasal inoculation of vaccinia were carried out in parallel with those on immunity following intravenous injections, the same preparations of vaccinia and ectromelia virus being used in both experiments. Table 3 shows the results, which resemble rather closely those shown in Table 1, although the higher dilutions were less effective when inoculated intranasally than when injected intravenously. The relationship between titre of V-AHA and death from challenge with the "Moscow" strain of ectromelia seen after intravenous injections is not evident after the intranasal inoculation of the lower dilutions of vaccinia. As before, in only two out of the ten mice dying after the inoculation of "Moscow" strain virus in groups in which some mice survived, was life prolonged beyond the normal incubation period of the disease.

Intraperitoneal Inoculation of Vaccinia Virus.

Five and six days after the intraperitoneal inoculation of a strain of neurovaccinia adapted to mouse-brain, Eyer (1937) found a slight viscous peritoneal exudate, and the spleen was enlarged, engorged and the follicles were hyperplastic. A clear pleural exudate and small foci of haemorrhage and consolidation in the lungs were commonly seen. Perivascular round-cell infiltration occurred most commonly in the neighbourhood of the interlobular veins of the liver, although Eyer was uncertain whether this change was due to vaccinia. Occasionally perivascular round-cell infiltration and small areas of necrosis were seen in the testis. All these organs contained vaccinia virus.

No such changes followed the intraperitoneal inoculation of "Gillard" vaccinia virus. The only abnormality seen was slight oedema of the tail, sometimes accompanied by very slight scabbing, best seen six days after the inoculation. Inoculation of suspensions of various organs on the chorioallantois after the intraperitoneal inoculation of undiluted vaccinia virus failed to reveal virus in any material except the suspension prepared from the tail (Table 5). Immersion of the tail in hot water, similar to that used before the intravenous inoculation of mice, was not followed by any increase in the severity of the tail lesions, or their virus content.

The antibody response was much weaker than that following intravenous or intranasal inoculation, the V-AHA titre varying from 20 to 160 (Geometric mean 70 $\pm$ 14).

The results of challenge inoculation of mice which had been inoculated intraperitoneally a fortnight earlier with various dilutions of vaccinia virus are shown in Table 4. Some protection is given against "Hampstead" ectromelia with dilutions up to 1:10,000, but even after the inoculation of undiluted vaccinia virus, a number of mice died on challenge within the normal

TABLE 3.

Immunization of mice against ectromelia with living "Gillard" vaccinia virus (chorioallantoic titre $2{\cdot}8 \times 10^8$ infective particles per ml.) 0·05 ml. inoculated intranasally.

Dilution of vaccinia virus.		Challenge with ectromelia virus 1: 10, 0·2 ml. by intraperitoneal inoculation. "Hampstead" strain ($2{\cdot}8 \times 10^6$ infective particles per ml.).							"Moscow" strain. ($3{\cdot}7 \times 10^8$ infective particles per ml.).												V-AHA titre. Geometric mean ± S.E.
10-6	V-AHA before challenge	5			<5	<5			5	<5	<5										90±70
	Result of challenge	5	5	5	7	13	±		4	4	4	4	4	4							
10-5	V-AHA before challenge	<5	<5				5		5	<5	0										
	Result of challenge	5	5	5	5	6	+		4	4	4	4	4	6							
10-4	V-AHA before challenge	160	80	320						<5	200		320								
	Result of challenge	+	+	±	±	—	—		4	5	±	±	—	—							
10-3	V-AHA before challenge			200	160	640			320	80		320									230±70
	Result of challenge	±	±	±	±	—	—		4	5	9	±	±	±	±						
10-2	V-AHA before challenge	160	320	320	500	320	320	160	640	320	160	160	160	160	640	640	640	200	160	160	280±36
	Result of challenge	+	±	±	—	—	—	—	4	4	+	±	±	±	—	—	—	—	—	—	
10-1	V-AHA before challenge	640	1,280	1,280	640	400	320		640	640	400	400	400	400	320	160	400	400	320	320	460±54
	Result of challenge	±	—	—	—	—	—		4	±	±	±	±	±	±	±	—	—	—	—	
Undiluted	V-AHA before challenge	640	1,280	640	320	200	160		400		1,280	640	1,000	320	320	320	200				440±80
	Result of challenge	±	—	—	—	—	—		5	7	±	±	—	—	—	—	—				
Controls		5	5	5	5	5	5	6	9	12					3	4	4	4	4	4	5	5	5	

Symbols as in Table 1.

TABLE 4.

Immunization of mice against ectromelia with living "Gillard" vaccinia virus (chorioallantoic titre—1·1 × 10⁸ infective particles per ml.) inoculated intraperitoneally, subcutaneously, and by scarification.

Mice immunized with vaccinia virus.		Challenge with ectromelia virus 1:10, 0·2 ml. by intraperitoneal inoculation. "Hampstead" strain (1·3 ×10³ infective particles per ml.)	"Moscow" strain (1·2×10⁸ infective particles per ml.)	V-AHA titre. Geometric mean ± S.E.
By intraperitoneal inoculation.				
10⁻⁶ 0·25 ml.	Result of challenge	5 5 5 10 10 +		
10⁻⁴ 0·25 ml.	Result of challenge	6 8 9 13 + +		
10⁻² 0·25 ml.	Result of challenge	6 8 11 + + +		
10⁻¹ 0·25 ml.	V-AHA before challenge	20 80 160 80 80 40 †	20 160 40 160 80 80	67±14
	Result of challenge	4 4 — — — — —	3 3 4 4 5 7	
Undiluted 0·25 ml.	V-AHA before challenge		80 80 60 40 20 20 160 160 20 160 80 160 100 100 100	70±14
	Result of challenge		3 4 4 4 4 7 + + + ± ± — — — —	
By subcutaneous inoculation.				
10⁻¹ 0·2 ml.	V-AHA before challenge	5 10 10 15 20 20 20	40 15 20 20 80 20	18± 3
	Result of challenge	4 4 4 5 + ± —	3 4 4 4 4 —	
Undiluted.	V-AHA before challenge	5 20 5 40 80 200		23±15
	Result of challenge	10 + ± ± ± —		
By scarification.				
vaccinia virus undiluted	V-AHA before challenge	20 320 100 60 50 30	80 60 40 40 40 40 40 40 40 40 40 40 40 20 160 80	50± 7
	Result of challenge	4 ± — — — — —	3 4 4 10 + ± ± ± ± ± ± ± ± — —	
Controls.		4 4 4 4 6 6 6 6 6 6 7	3 4 4 4 4 6	

Symbols as in Table 1.

incubation period of ectromelia. All mice inoculated with 1:10 vaccinia and challenged with "Moscow" ectromelia died within the normal incubation period.

Subcutaneous Inoculation of Vaccinia Virus.

Eyer (1937) found that virus could be isolated from the lung and liver but not from other organs after subcutaneous inoculation of mice with neurovaccinia adapted to mouse-brain. We found that the subcutaneous inoculation of an undiluted suspension of virus caused no sickness and was followed by pathological changes only in the draining lymph nodes. Virus was isolated from the right inguinal node on the fourth and fifth days after the subcutaneous inoculation of a 1:10 dilution of the virus above the right groin and from subcutaneous tissue in the right groin, but from no other organs (Table 5). The V-AHA titres obtained after 1:10 virus were low (Geometric mean = 18±3) and the protection conferred against challenge inoculation with ectromelia virus was likewise slight (Table 4). Inoculation of undiluted vaccinia virus subcutaneously was followed by a somewhat higher degree of protection against "Hampstead" ectromelia, but it was poor compared with the results following intravenous and intranasal inoculations or scarification.

TABLE 5.

Presence of vaccinia virus in various organs of mice at any time within six days after inoculation by various routes.

	Mode of inoculation.							
	Intravenous.		Intraperitoneal.		Subcutaneous.		Scarification.	
Organ.	"Gillard."	"Neuro-vaccine."*	"Gillard."	"Neuro-vaccine."	"Gillard."	"Neuro-vaccine."	"Gillard."	"Neuro-vaccine."
Blood	+‖	0	0	0	0	0	0	0
Lymph nodes	+¶	0	0	0	+	0	+	+
Spleen	+	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0
Lungs	+	++	0	+	0	0	0	0
Testis	..	0	0	0	..	0	..	0
Tail	+++	0	+	0	0	0	..	..
Skin	..	..	..	..	..	..	+++	+++
Subcutaneous tissue	..	..	..	..	+	++	..	..

* "Neurovaccine" animals tested on fifth day only.

¶ + to +++ indicate varying amounts of virus present.

‖ Virus found in blood only during the first twenty-four hours.

Scarification with Vaccinia Virus.

Rosenau and Andervont (1931) investigated the response of mice to scarification with several strains of vaccinia virus. They were unable to produce lesions on the shaved skin of the abdominal wall, but found that vesiculation and scabbing irregularly followed scarification of the pad of the foot or the base of the tail. In spite of the failure to produce lesions on the abdominal wall, immunity to subsequent pad scarification or intracerebral inoculation of vaccinia virus was produced in a large proportion of animals tested.

By applying glycerolated, undiluted "Gillard" virus to scarification of the shaved abdominal wall of mice we obtained vesiculation and scabbing in all treated animals. The reaction reached its maximum development on the sixth day. No abnormality was seen in the internal organs of animals killed at various intervals after scarification, but the inguinal lymph nodes were usually enlarged and engorged. Large amounts of virus were isolated from suspensions of the scarified skin, and the inguinal lymph nodes also contained small amounts of virus between the third and sixth day. Virus was not isolated from the other organs (Table 5).

The antibody response was rather low, the V-AHA titre varying between 20 and 320 (Geometric mean = 50±7).

Scarification conferred a fairly high degree of protection against both strains of ectromelia virus, although deaths within the normal incubation period occurred in both groups in the individuals with the lowest V-AHA titres (Table 4). Although V-AHA titres following the intraperitoneal inoculation of 1:10 virus or scarification with undiluted virus were

TABLE 6.

Effect of intranasal instillation of undiluted suspensions of several strains of vaccinia virus.

Strain of virus.	Chorio-allantoic titre (infective particles per ml.)		Mouse killed six days after inoculation.																				
																	Lungs.				Serum.		
																	Consoli-dation.	Chorio-allantoic titre.	V-HA titre‡.	V-AHA titre.			
"Neuro-vaccine"	4×10⁷	Result	4*	5	5	5	5	6	6	6	6	8	+++‖	—	—	+++	1×10⁷	0					
		V-AHA titre†											320	320	320								
"CSL"	2×10⁷	Result	++	++	+	+	+	+	—	—	—	—	—			++	8×10²	0	10				
		V-AHA titre	400	320	640	320	320	160	320	320	320	200	200										
"Java"	1×10⁷	Result	4	+	+	+	+	+	—	—	—	—	—			++	2×10³	0	10				
		V-AHA titre		640	640	200	160	160	400	400	320	320	200										
"Gillard"	2×10⁸	Result	++	+	+	+	+									++	2×10⁵	0	10				
		V-AHA titre	1,280	640	640	640	400																

* 4, 5, etc. = death with extensive consolidation of lungs 4, 5, etc. days after inoculation of virus.

‖ —, +, ++, +++ = no, slight, moderate and extensive consolidation of lungs at post-mortem 14 days after inoculation.

† V-AHA titre = vaccinia antihaemagglutinin titre determined with four haemagglutinating doses of "Gillard" vaccinia tested 14 days after inoculation.

‡ V-HA titre = vaccinia haemagglutinin titre determined with 2 p.c. fowl cells.

similar, multiplication of the vaccinia virus was much greater after scarification, and protection, especially against the more virulent "Moscow" strain, much more effective.

Comparison of the Effect of Different Strains of Vaccinia Virus on Mice.

The different results reported by Lush (1940) and Nelson (1938) after the intranasal inoculation of mice with vaccinia virus, and the greater severity of lesions occurring after the inoculation by various routes of neurovaccinia adapted to mouse-brain (Eyer, 1937) led us to carry out short comparative experiments using several strains of vaccinia virus.

Undiluted suspensions of four strains of virus of about the same titre were inoculated intranasally in eight-weeks-old mice. The results are shown in Table 6. Tested by intranasal inoculation, neurovaccinia is evidently much more virulent than any of the other (dermal) strains used, and the latter vary slightly amongst themselves.

In each group the animal killed on the sixth day showed fairly extensive consolidation of the lungs, although the virus content of the lung in the "Neurovaccine" and "Gillard" groups was much higher than in the other two groups.

Surviving mice develop V-AHA to about the same titre after infection with each of the strains used. V-AHA titres of individual sera from survivors of the "Neurovaccine" or "Gillard" groups were the same, using either of these two strains of vaccinia as haemagglutinin.

In this limited experiment, neurovaccinia appeared to be comparable in virulence to the strain of vaccinia used by Nelson. None of the dermal strains used appears to be of such low virulence as the Lister lapine used by Lush.

It appeared likely that neurovaccinia might give results after inoculation by various routes similar to those obtained by Eyer (1937) and small groups of mice were inoculated with neurovaccinia intraperitoneally, intravenously, subcutaneously and by scarification. Five days later two mice of each group were killed and examined post-mortem, and the organs pooled, ground, and the suspension inoculated on the chorioallantois. The results are indicated in Table 5. Lesions of the tail were not seen after the intravenous inoculation, and although a suspension of skin from the area of scarification contained much virus the skin lesions were slight. On the other hand virus was found in the lungs after both intravenous and intraperitoneal inoculation of "Neurovaccine." The post-mortem lesions were not as severe as Eyer had found with neurovaccinia adapted to mouse-brain and the virus was by no means as widely distributed in the organs. However, "Neurovaccine" appears to have an affinity for lung tissue and "Gillard" vaccinia for the skin of the mouse.

Duration of Immunity.

An experiment was undertaken to determine the duration of immunity to challenge doses of "Hampstead" ectromelia of various strengths at intervals of two to twenty weeks after the intravenous inoculation of 1:100 vaccinia virus. A very slight diminution in immunity was found (Table 7). Unfortunately several mice kept for challenge inoculation after twenty weeks died from pneumonia due to a Friedländer-like bacillus, and their cage mates were destroyed, so that later tests could not be made. V-AHA titres measured two and twenty weeks after the initial inoculation of vaccinia virus showed a considerable fall in antibody level (at two weeks: 320, 320, 160, 160, 80, 80, 80, 80, 40, 10. Geometric mean = 90±30; at twenty weeks: 20, 20, 15, 15, 15, 10, 10, 5, 5, 5, 5, 5. Geometric mean = 9±1·6).

TABLE 7.

Immunity to "Hampstead" ectromelia virus inoculated intraperitoneally at intervals after intravenous inoculation of vaccinia virus 1:100 (chorioallantoic titre $7{\cdot}2 \times 10^8$ infective particles per ml.).

Interval between inoculations.	V-AHA geometric mean ± S.E.	Dose of ectromelia virus.	Results of challenge. Immunized mice.	Control mice.
2 weeks	90±30	10^{-5}		+ + + + + +
		10^{-4}		7 7 + + +
		10^{-3}	— — — — — —	5 6 6 7 + +
		10^{-2}	± ± — — — —	
		10^{-1}	5 ± — — — —	
20 weeks	9±1·6	10^{-5}		6 6 8 + + +
		10^{-4}		7 7 8 9 + +
		10^{-3}	± — — — — —	6 6 7 8 10 +
		10^{-2}	5 5 ± ± ± ±	
		10^{-1}	± ± ± — —	

Symbols as in Table 1.

Nelson (1938) tested some mice which had survived intranasal inoculation of his virulent strain of vaccinia virus with a second inoculation a year later. Of four such mice, which had been subjected to mortuary selection as well as immunization, one died of vaccinial pneumonia, two suffered mild attacks of pneumonia and one remained unaffected. Clearly the immunity, which was complete two weeks after the inoculation, had declined somewhat after a year.

Time Required for the Development of Immunity.

Groups of six eight-weeks-old mice were inoculated intranasally with 1:100 dilution of "Gillard" vaccinia virus at intervals of two days. Fourteen days after the inoculation of the first group all mice were bled from the tail and the V-AHA titre of the serum determined. They were then challenged with "Hampstead" ectromelia virus, 1:10 dilution, inoculated intraperitoneally. Survivors were killed and examined fourteen days later.

The results obtained in the groups inoculated ten and twelve days after the instillation of vaccinia virus were identical with those of the fourteen-day group, and have been omitted from Table 8.

TABLE 8.

Development of immunity to "Hampstead" ectromelia virus at intervals after the intranasal inoculation of vaccinia virus 1:100 (chorioallantoic titre 1×10^8 infective particles per ml.).

Interval between vaccinia and ectromelia.		Challenge with "Hampstead" strain ectromelia virus ($2{\cdot}8 \times 10^8$ infective particles per ml.) 1: 10, 0·2 ml. by intraperitoneal inoculation.					
1 day	V-AHA before challenge						
	Result of challenge	5	5	5	5	5	5
3 days	V-AHA before challenge	0	0	0	0	0	0
	Result of challenge	4	5	5	5	6	+
4 days	V-AHA before challenge	0	0	0	0	0	0
	Result of challenge	5	5	+	±	±	±
6 days	V-AHA before challenge		10	10	5	<5	
	Result of challenge	±	±	—	—	—	
8 days	V-AHA before challenge	160	80	60	40	40	40
	Result of challenge	—	—	—	—	—	—
10 days	V-AHA before challenge	160	160	100	80	80	
	Result of challenge	—	—	—	—	—	—
14 days	V-AHA before challenge	160	160	120	120	80	60
	Result of challenge	—	—	—	—	—	—

Symbols as in Table 1.

V-AHA was first detected six days after inoculation of vaccinia virus and the titre rapidly increased to a maximum at ten days. The protection conferred in the four- and six-day groups was greater than would be expected from the V-AHA titres at those times, but the antibody level was rising.

There was no protection when inoculation of vaccinia preceded that of ectromelia by less than three days. No evidence has been obtained of interference between ectromelia and vaccinia viruses in the mouse, although concentrated suspensions of the viruses have been given simultaneously, or at intervals of a few minutes to an hour, both intravenously or by intravenous and intraperitoneal routes.

Effect of Age of Mice upon Immunity Conferred by Intranasal Inoculation.

Seven-day-old mice are much more susceptible to intranasal vaccinia virus than are older animals. Even when 0·01 ml. of 1:10,000 "Gillard" vaccinia virus suspension was instilled intranasally most animals were sick from the seventh to eleventh days after inoculation, with ruffled hair, respiratory symptoms, and sometimes conjunctivitis. With lower dilutions, the disease was more severe and deaths occurred in a number of animals. The growth of survivors was greatly retarded. The resistance of fourteen-day-old mice was much greater. With 1:100 vaccinia virus these mice showed slightly ruffled hair about the seventh and eighth days but were otherwise well and their growth was normal. Three-weeks-old mice behaved in exactly the same way as did eight-weeks-old mice, that is, they were unaffected by anything except an undiluted virus suspension.

An attempt was made to compare the antibody response (V-AHA) and protection

TABLE 9.

Effect of age of mice upon the immunity to ectromelia conferred by the intranasal inoculation of vaccinia virus (chorioallantoic titre 4×10^8 infective particles per ml.).

Age of mice.	Dosage of vaccinia virus.		Challenge with "Hampstead" ectromelia virus (chorioallantoic $1{\cdot}2 \times 10^8$ infective particles per ml.), 1:10 by intraperitoneal inoculation, 14 days after vaccinia.	Serum V-AHA titre. Geometric mean ± S.E.
7 days	0·01 ml. 10^{-4}	V-AHA titre before challenge	640 320 320 200 160 160	260±60
		Result of challenge	— — — — — — — — — — — —	
	Controls (21 days old)		5 5 5 6 6 7	
21 days	0·03 ml. 10^{-2}	V-AHA titre before challenge	200 320 320 200 200 160 160 160 160 80 80 80 80 80 80	140±18
		Result of challenge	+ — — — — — — — — — — — — —	
	Controls (35 days old)		5 6 6 6 7 7	
8 weeks	0·05 ml. 10^{-2}	V-AHA titre before challenge	200 160 160 160 160 160	170± 6
		Result of challenge	± — — — — —	
	Controls (10 weeks old)		5 6 6 7 9	
10 months	0·05 ml. 10^{-2}	V-AHA titre before challenge	80 80 320 640 400 160 80 320 320 160 160 160	190±38
		Result of challenge	4 12 + ± ± ± ± — — — — —	
	Controls (10 months old)		5 6 6 6 6 6	

Symbols as in Table 1.

against ectromelia in mice of different ages. Seven-day-old mice were inoculated intranasally with 0·01 ml. 1:10,000 vaccinia virus, twentyone-day-old mice with 0·03 ml. 1:100 virus, and eight-weeks-old and ten-months-old mice with 0·05 ml. 1:100 virus. V-AHA titres were determined fourteen days later, just before challenge inoculation with 1:10 "Hampstead" ectromelia virus. With the smaller mice (seven days old when inoculated) several mice were killed and the V-AHA titre of the blood of these animals determined, as the animals were too small for effective bleeding from the tail veins.

The results are shown in Table 9. Mice of all the ages tested developed V-AHA to about the same titre, and all showed a high degree of protection against subsequent challenge with ectromelia virus. The ten-months-old mice were slightly more susceptible to ectromelia than those of other groups.

DISCUSSION.

The extensive laboratory manipulations to which vaccinia virus has been subjected have resulted in the production of a large number of strains which differ slightly or considerably in several characters. As the investigations of Levaditi and Nicolau (1923), Rosenau and Andervont (1931), Eyer (1937), Nelson (1938), Iguchi (1939) and Lush (1940), and our own experiments have shown, the virulence of vaccinia for mice is one such variable character. Neurovaccinia, whether adapted to mice or not, appears to be more virulent than most strains of dermal vaccinia especially after intranasal inoculation.

In recent years considerable interest has been taken in the effect of age on the susceptibility of mice to infection with animal-passaged strains of neurotropic

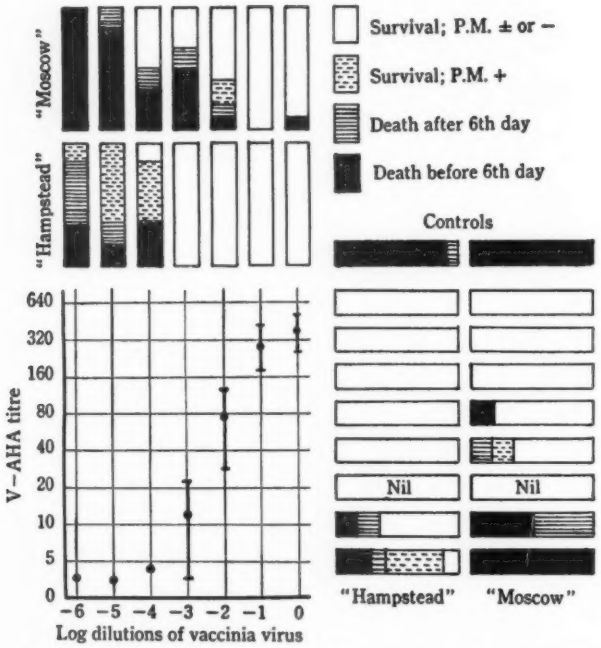

Fig. 1. Geometric mean V-AHA titres (± twice their standard errors; and results of challenge inoculations of "Hampstead" and "Moscow" ectromelia virus, correlated with dose of vaccinia and with V-AHA titres attained fourteen days after the intravenous inoculation of various dilutions of "Gillard" vaccinia virus. Data taken from Table 1.

viruses, the common finding being that age has no effect on susceptibility to intracerebral inoculation of these viruses, but that only young mice (less than 15 days old) are susceptible to peripheral inoculation (Morgan, 1941; Lennette and Koprowski, 1944). Not much work has been reported on age variations of susceptibility to other types of virus. We found that seven-day-old mice were more susceptible than older mice to the intranasal inoculation of vaccinia virus, but that this susceptibility had greatly decreased by the fourteenth and disappeared by the twenty-first day.

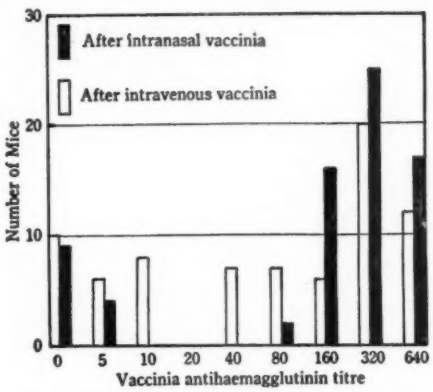

Fig. 2. Frequency of titres of V-AHA after intranasal and intravenous inoculation of mice with various dilutions of vaccinia virus.

Most workers who have investigated the influence of age on immunizability (e.g. Baumgartner, 1934a; Casals, 1940; and Morgan, 1941) conclude that adult animals are more readily immunizable than young ones. Baumgartner (1934b and 1934c) also found qualitative differences in the antisera, even when antibody titres were identical. Antisera from young animals were less "avid" than those from adult animals. Casals (1940) reported that neutralizing antibodies against rabies (fixed virus) were produced equally well by seven-day-old, twenty-day-old and sixty-day-old mice, but the titre of immunity to intramuscular challenge inoculation was much higher in the older mice than in the seven-day-old animals. Our experiments were not elaborate enough to detect small differences in protection, but they show that after intranasal infection with vaccinia virus seven-day-old mice develop V-AHA to the same high titre as do older animals, and they resist intraperitoneal challenge inoculation with "Hampstead" ectromelia virus equally well.

The protection conferred against ectromelia by previous infection of mice with vaccinia virus is due to the immunological response of the animals to the first infection, and the interference phenomenon appears to play no part in it. No effect appears until the interval between infections is four days, and thereafter the degree of protection rapidly increases to reach a maximum when eight days intervene. Although the V-AHA titre falls considerably when the interval is prolonged to twenty weeks, the immunity decreases only slightly.

It is reasonable to regard the antihaemagglutinin titre at the conventional period of fourteen days as a useful index of the overall intensity of the immunity response to the particular antigenic stimulus provided. There is no evidence for or against the assumption that the V-AHA is directly responsible for part of the observed immunity but the persistence of immunity after the titre has fallen to about a tenth of its peak value indicates that other factors are also and probably more importantly concerned.

Both the antibody response and immunity appear to be closely related to the intensity and duration of multiplication of the vaccinia virus. The minimum infecting dose is small and multiplication of the virus considerable after intravenous and intranasal inoculation and scarification, and these methods produce the most effective immunity.

Fig. 1 shows graphically the relationship between V-AHA titre and the result of challenge inoculation with ectromelia and the dilution of vaccinia virus used for intravenous infection of mice and the result of the challenge inoculation. The geometric mean V-AHA titres after inoculation of various dilutions of vaccinia virus are shown by points on the graph and plus and minus twice the standard deviations indicated. The correlations between dose of vaccinia virus, V-AHA titre, and immunity to ectromelia are clearly shown. Protection against "Moscow" ectromelia virus is seen to be much less effective than against the "Hampstead" strain.

In Fig. 2 the V-AHA titres following intranasal and intravenous inoculation of vaccinia virus are grouped according to the frequency with which various titres were found. After intranasal inoculation the titre is either very low (≤ 5) or high ($\geqslant 160$), whilst after intravenous inoculation the response is fairly evenly distributed. Presumably, if an infecting dose of virus is deposited on the large area of respiratory epithelium, considerable multiplication will occur with consequent high antibody production. After intravenous inoculation, on the other hand, there is no such large susceptible area available and all grades of multiplication and therefore of antibody production occur.

TABLE 10.

V-AHA titres after vaccination with "Gillard" vaccinia by various methods and fate of mice after challenge with "Moscow" ectromelia virus.

	Mode of vaccination.			
V-AHA titre.	Intravenous.	Scarification.	Intranasal.	Intraperitoneal.
5– 20	4/5*	1/2	3/3	3/5
40–100	1/11	3/17	1/1	7/11
160–400	0/14	0/3	4/28	2/6
$\geqslant 640$	0/9		2/9	

Numerator = number of mice dying within usual incubation period of ectromelia.
Denominator = number of mice in group.

In Table 10 the fate of mice vaccinated by intravenous, intranasal and intraperitoneal routes and by scarification, and inoculated a fortnight later with "Moscow" ectromelia virus is analysed according to V-AHA titre found prior to the challenge inoculation. Animals with titres less than 5 are omitted. When the titre was low (5–20) the fate was the same in each group, death within the usual incubation period occurring in the majority of instances. When the V-AHA titre was moderate or high (40 and higher), the fate of mice depended significantly on the route by which the immunizing infection had been induced. In order of diminishing effectiveness are the routes intravenous, scarification, intranasal, intraperitoneal, but only the last fulfils the usual statistical criteria for significance. [Comparing the figures after intraperitoneal inoculation in turn with those for intravenous and intranasal inoculation and scarification $\chi^2=15 (P<\cdot001)$, $\chi^2=6\cdot8 (P=\cdot01)$, $\chi^2=4\cdot4 (P=\cdot04)$ respectively.]

The anomalous result recorded after intraperitoneal vaccination and intraperitoneal challenge may be related to the phenomenon described by Koprowski and Cox (1946). Working with Japanese B encephalitis in mice, they found that the same vaccine showed varying immunizing capacity depending primarily on the route of inoculation used for immunization and challenge. In particular, chick-embryo vaccines were deficient in immunogenic capacity when immunization and challenge were both by the intraperitoneal route.

The usual method of natural infection of mice with ectromelia is through minute abrasions of the skin (Fenner, 1947), and it was therefore thought desirable to use a method of immunization which would not produce vaccinial lesions of the skin, so that experiments involving natural spread in immunized and normal mice would not be complicated by this factor. In animals 14 days old or older, intranasal inoculation with 1 : 100 "Gillard" vaccinia virus (or any other of several dermal strains) produces no ill effects in the mouse, is easy to perform, and produces a high degree of immunity to ectromelia. It has been adopted as the standard technique.

It has not been possible to produce complete protection of all inoculated mice against intraperitoneal inoculation of "Moscow" ectromelia by immunization with vaccinia virus. It remains to be determined whether immunization will completely protect animals against natural infection with this strain.

SUMMARY.

Mice can be infected with "Gillard" vaccinia virus inoculated by a variety of routes. Local symptoms and skin lesions only occur after scarification, intravenous and intraperitoneal inoculation of concentrated suspensions. Intranasal inoculation of concentrated suspensions causes sickness (coryza and pneumonia) with a low mortality in adult mice, but more dilute suspensions cause no symptoms. Suckling mice are much more susceptible to intranasal inoculations, pneumonic symptoms occurring with dilutions up to 1 : 10,000. Several other strains of dermal vaccinia tested by intranasal inoculation gave results similar to those found with "Gillard" strain.

Neurovaccinia virus causes a high mortality on intranasal inoculation in adult mice.

Antibody (vaccinia antihaemagglutinin) can be detected within six days of intranasal infection with vaccinia virus and reaches its maximum titre by the tenth day. The titre falls to a low level by the twentieth week after infection. Methods of inoculation which are followed by considerable multiplication of the virus cause maximum production of vaccinia antihaemagglutinin. Young mice (7 days old) infected intranasally with vaccinia virus produced as much antihaemagglutinin and exhibited as high an immunity to challenge inoculation with ectromelia virus as did adult mice.

The vaccinia antihaemagglutinin titre estimated a fortnight after inoculation with vaccinia virus is a useful index of the immunity of mice to subsequent intraperitoneal inoculation of ectromelia virus. The relationship is less evident after intraperitoneal inoculation of vaccinia than after infection by other routes. The "Moscow" strain of ectromelia virus is more virulent than the "Hampstead" strain and no method of immunization with vaccinia virus produced complete immunity to it.

Intranasal inoculation with diluted vaccinia virus, which gives a high degree of immunity, causes no symptoms, and can be carried out rapidly on a large scale, is the method of choice for immunizing mice against ectromelia.

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STUDIES IN INFECTIOUS ECTROMELIA IN MICE

II. NATURAL TRANSMISSION: THE PORTAL OF ENTRY OF THE VIRUS.

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Although ectromelia has been used for the experimental study of the epidemiology of virus diseases (Greenwood *et al.*, 1936), there is no agreement on its mode of spread.

In her original description of the disease Marchal (1930) reported some preliminary experiments on transmission by feeding infected viscera and by placing normal mice in contact with infected animals under ordinary laboratory conditions. Results were irregular and no conclusions on the mode of transmission were reached. Subsequent workers (McGaughey and Whitehead, 1933; Schoen, 1938; Kikuth and Gönnert, 1940) reported the occurrence of ectromelia in laboratory stocks of mice in England, France and Germany, but offered no explanation of the natural mode of spread.

Edward, Elford and Laidlaw (1943), reporting that they had successfully infected mice by placing them in an atmosphere in which a suspension of ectromelia virus had been atomized, stated that such exposure caused characteristic lesions of the liver and spleen and an extensive pneumonia. They considered that the post-mortem appearance of infected animals was closely analogous to "naturally occurring airborne infection."

Greenwood *et al.* (1936) do not discuss the mode of spread of the disease but in the course of the discussion on the epidemics of ectromelia assume that it is a droplet infection (p. 92).

It was thought desirable to determine the usual mode of spread of the disease before embarking on experiments involving the natural spread of ectromelia through large colonies of mice. In this paper the portal of entry of the virus is discussed, and in a subsequent paper the portals of exit of the virus and the duration of infectivity of infected mice will be described.

MATERIAL AND METHODS.

The material and methods used have been described previously (Fenner, 1947).

Marchal's "Hampstead" strain and the "Moscow" strain of ectromelia virus were used, stock suspensions being prepared from infected chorioallantoic membranes.

Infection of mice was judged by post-mortem findings, and in animals in which there was minimal or no macroscopic evidence of infection at post-mortem, the ectromelia antihaemagglutinin (E-AHA) titre of the serum was determined. We have never encountered mouse sera which cause nonspecific inhibition of haemagglutination by ectromelia virus, and this test enabled subclinical infections which also failed to produce macroscopic lesions of the viscera to be detected.

Mice infected with "Moscow" ectromelia virus were housed in a different room from the other experimental animals.

EXPERIMENTAL RESULTS.

Mice were infected with ectromelia by a variety of methods and the post-mortem changes compared with those which follow the natural disease. Previous writers have sometimes failed to remark upon the different post-mortem appearances which follow different methods of

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inoculation. Dingle (1941), for example, in his description of the pathology of the natural disease, mentions increased peritoneal, pericardial and pleural fluids in mice dying acutely, and ascites, hydrothorax and fat necrosis in the chronic type of infection. We have never found fat necrosis except after intraperitoneal inoculation of the virus, and abundant pleural or peritoneal exudates occur almost invariably after intraperitoneal inoculations but very rarely in the natural disease.

The possible natural means of entry of the virus are by feeding, through the respiratory tract, through the skin or superficial mucous membranes, through the placenta, or by means of an insect vector. In Table 1 the 50 p.c. endpoints for infection of mice by different routes are given for the two strains of virus used. Specific death, specific post-mortem lesions at the end of the experiment, or the presence of E-AHA in the serum were taken as evidence of infection.

TABLE 1

50 p.c. endpoints (for infection) after inoculation of mice by various routes.

Mode of inoculation.	Oral.	Intranasal with anaesthetic.	Intranasal without anaesthetic.	Pad inoculation.	Pad scarification.	Intraperitoneal inoculation.	Chorioallantoic membrane (egg).
Dose (ml.)	0·05	0·05 ml.	0·01 ml.	0·03 ml.		0·05 ml.	0·05 ml.
"Hampstead" strain	10^{-1}	$10^{-7}+$*	10^{-4}	$10^{-7}+$	$10^{-4.5}$	10^{-8}	10^{-7}
"Moscow" strain	10^{-2}	10^{-8}	10^{-4}	$10^{-8}+$	$10^{-5.5}$	$10^{-8}+$	10^{-7}

*$10^{-x}+$ indicates that the 50 p.c. endpoint was not reached at that dilution, the highest tested.

Infection by an Insect Vector.

Marchal (1930) suspected at one stage that bugs might be concerned in the transmission of ectromelia, but this was soon disproved. Our mice are free of ectoparasites, and we have obtained no evidence suggesting that ectromelia may be a disease transmitted by arthropods.

Infection by Feeding.

Animals were deprived of food and water overnight and next morning induced to drink 0·05 ml. of various dilutions of virus from a Pasteur pipette. Only animals which had received very large doses of virus were infected (Table 1). A few of these had primary ectromelia lesions of the snout caused by injury with the pipette during feeding, but most showed no local lesions of the skin or of the gastro-intestinal tract. The post-mortem findings resembled those of the natural disease.

Experiments were carried out to determine whether young mice might be infected by the ingestion of milk from infected mothers. When female mice which had just borne their young, or pregnant mice which bore young one, three and five days later, were inoculated intradermally with ectromelia virus the nursling mice almost invariably died of acute ectromelia about a fortnight after the inoculation. No skin lesions were found on these young mice.

Milk was collected from the stomachs of young mice killed five, six, seven and eight days after intradermal inoculation of their mothers and subinoculation revealed a small amount of virus in one specimen collected on the seventh day, and in both tested on the eighth day.

The evidence available is insufficient to allow a definite decision to be made on the mode of infection of nursling mice. The skin of such young animals fails to react to virus rubbed on in the manner described later in this paper and the treated animal dies of acute ectromelia without the development of a local skin lesion. At the time when virus was detected in the mother's milk, large amounts were also being excreted from skin lesions, and probably in the faeces and saliva also.

In any case, infection of nurslings by the mother's milk could only be a minor method of natural transmission, for like other mice, mothers are infective only during the acute stage of the disease, from the seventh to the fourteenth, and occasionally the twenty-first day after infection. Females which have recovered from an attack of ectromelia do not infect their young.

Infection by Inhalation.

Kikuth and Gönnert (1940) have described the extensive pneumonia which follows intranasal inoculation of ectromelia virus. In our experience this picture of pneumonia with little change in the abdominal viscera was by no means the rule. It was found only under two circumstances, (1) when normal mice were given very concentrated virus suspensions intranasally, and (2) when mice which had recovered from previous infection with ectromelia succumbed to subsequent intranasal inoculation. When the virus suspension was more dilute, patchy congestion and

consolidation occurred in the lungs of normal mice, but characteristic changes were always seen in the liver and spleen. The pneumonia which follows the inhalation of a fluid suspension or an atomized mist of ectromelia virus (Edward, Elford and Laidlaw, 1943) is never seen in the natural disease, and this fact is sufficient to exclude the lower respiratory tract as a natural portal of entry.

It was thought possible that infection might occur naturally through the nasal mucosa. To simulate this, small amounts of virus suspension (0·01 ml.) were instilled into the noses of mice without anaesthetization. Infection occurred when relatively concentrated virus suspensions (>10–4) were used, but the clinical picture in infected mice did not resemble that seen in the natural disease. The snout became very swollen, there were scabbed lesions on the nose, severe bilateral conjunctivitis occurred early in the disease, and the affected mouse sneezed and wiped its nose frequently with its fore paws. There was a slight nasal exudate.

Infection Through the Conjunctiva.

Conjunctivitis is a common feature of ectromelia, in the natural disease and after inoculations by a variety of routes. It occurred, for instance, in 15 out of 22 animals which survived for more than eight days after scarification of the sole of the foot with virus of the "Moscow" strain. It is rarely seen before the seventh day after inoculation. It commences as a thin, semipurulent discharge which is followed by gumming of the lids and when healing occurs by a loss of hair around the eye. It often starts on one or other side but later becomes bilateral, and it is not accompanied by much swelling of the skin and subcutaneous tissue around the eye.

A small drop of undiluted suspension of virus was instilled in one eye with a drawn-out Pasteur pipette, the eye opened and closed a few times and excess fluid removed. Infection followed in every case, with both strains of virus. With the "Moscow" strain a watery discharge commenced from the inoculated eye on the fourth day and the surrounding skin became very swollen and inflamed. Two mice died on the eighth day and the third animal developed generalized cutaneous lesions but survived. In no case was the other eye affected. With the "Hampstead" strain the local lesions followed a similar course, but as all animals survived it developed further and the skin around the affected eye was greatly swollen with a scab covering the eye itself. The other eye was unaffected in all cases. A picture similar to this is occasionally seen in the natural disease, especially if normal mice have eaten the body of a companion which has recently died of acute ectromelia. It is conceivable that under these circumstances, the conjunctiva may be soiled with virus from infected viscera.

Infection Through the Vagina.

Natural spread of ectromelia occurs just as readily when the animals under test are all the same sex as when sexes are mixed, so that sexual intercourse is unlikely to be an important mode of transfer of the infection. However, infection occurs readily through the vaginal mucous membrane, as was established by passing a small cotton-wool swab soaked in a suspension of virus into the vagina and then removing it. Inflammation, oedema and sometimes discharge from the vagina commenced three days later and continued until death if this occurred early, or otherwise healed by the fourteenth day.

Semen, removed from the seminal vesicles of five mice which had died of acute ectromelia, was suspended in saline and inoculated into healthy mice. There was a small amount of virus in two specimens. Sexual intercourse might, therefore, be an occasional mode of infection of female mice under natural conditions.

Intra-Uterine Infection.

Wylegschanin (1936) conducted experiments on the passage of ectromelia virus through the placenta of the mouse. He inoculated a number of pregnant female mice in the pad of the hind foot and scarified them at various periods (5–12 days) after inoculation. Various organs including the placentas, foetuses and amniotic fluid (if available) were tested for virus by subinoculation in mice. He found that the placenta almost invariably contained a large amount of virus, but in only two animals out of thirteen, killed 8 and 11 days respectively after inoculation, did the suspension of foetal tissues contain virus.

Some twenty pregnant mice have been inoculated with ectromelia, but no effect on the course of the pregnancy has been observed and the young born of such mice were not infected at birth. However, subinoculations in healthy mice of suspensions of foetus and placenta of a pregnant mouse which died eight days after inoculation of "Hampstead" ectromelia in the tail were positive, and characteristic inclusion bodies were seen in the subepithelial tissues of another foetus from this mother.

Two pregnant females were inoculated intravenously and died four and five days later. In each case the uterus was removed and washed three times in saline. The right horn and its contained foetuses were put aside and the foetuses removed from the left horn and washed twice in saline. The amniotic fluid was removed and the foetuses carefully separated from the placentas

and membranes. Various tissues were pooled, ground with sand and suspended in saline, and the supernatant after centrifugation inoculated in various dilutions on the chorioallantois. The results are shown in Table 2.

TABLE 2.

Occurrence of ectromelia virus in uterus, placenta, and foetuses of an infected female.

Material.	Foci*.	Dilution.
Left uterine horn plus foetuses	5	10^{-2}
Right uterine horn after removal of foetuses	250	10^{-1}
Pooled membranes and placentas of removed foetuses	40	10^{-1}
Pooled amniotic fluids	0	Undiluted
Four separate foetuses, each	0	Undiluted

*The number of ectromelia foci found on chorioallantoic membranes of embryos inoculated with the dilutions indicated.

It is certain that intra-uterine infection does not play a rôle in the natural history of ectromelia comparable to that which it plays in lymphocytic choriomeningitis in the mouse (Traub, 1939). However, Wylegschanin's evidence indicates that occasionally damage to the placenta is sufficiently severe to permit infection of the foetus, just as occurs rarely in human variola and vaccinia (Lynch, 1932).

Infection through the Skin.

The frequent occurrence of lesions of the feet led Marchal (1930) and other English workers to suspect that the virus might enter the body through the skin of the foot, but there was no confirmation of this idea. We found that if a swab soaked in a concentrated virus suspension was rubbed over the lightly scarified skin of the pad of the foot, the abdominal wall or any other part of the body, infection always occurred. Swelling and inflammation of the scarified area appeared on the fourth day and the lesion increased in size and became scabbed by the sixth day. Subsequently the mouse either died of acute ectromelia, suffered a generalized attack with widespread skin lesions, or recovered without obvious clinical extension beyond the local primary lesion. The post-mortem findings in fatal cases and in animals killed at various stages after recovery resembled those of the natural disease, except that the local primary skin lesion was usually more extensive.

Experiments were then carried out to determine whether infection could occur through the unbroken skin. Mice were placed in individual glass jars with cotton-wool bedding for five days prior to application of the virus, so that small abrasions received during cage life could heal. In half the test animals virus was rubbed gently on the skin of the abdominal wall with a cotton-wool swab, in the other animals a small drop of concentrated suspension was placed on the skin, very gently rubbed over an area of one square centimetre with a smooth glass rod and the excess fluid carefully removed. All animals whose skin was rubbed with a soaked swab developed local lesions in that area on the seventh day, and if the animals survived these increased considerably in size and became covered with a large scab, under which healing occurred and was complete by the fourteenth day. When the virus suspension was placed in contact with the skin with a minimum of trauma infection did not occur.

Marchal (1930) named the disease "ectromelia" because of the frequency with which amputation of an extremity occurred. In only ten out of several hundred cases have we seen amputation follow either natural infection or pad inoculation in adult mice, and Greenwood's (1936) experience is similar. In adult mice the skin of the pad of the hind foot is quite thick, and we were unable to infect animals by rubbing virus on the hind pad with a cotton-wool swab, whereas if the same swab was rubbed on the skin of the belly wall, infection invariably occurred. In an experiment in which pad inoculation was carried out on young (13 days to 21 days old) mice, amputation of the extremity was common. It is perhaps significant that Marchal's first observations were made on young mice just weaned and caged together. In such mice the pad of the foot is still relatively thin and primary lesions of the foot might, therefore, be not uncommon, and the leg is so thin that a severe lesion of the foot would cause amputation.

Infection from Contaminated Bedding.

The previous experiments indicated that infection could occur through the skin if slight trauma occurred. To see whether normal mice running in a cage in which the sawdust bedding was contaminated with virus would contract the disease, two ml. of a 1:10 suspension of virus ("Moscow" strain) were sprayed on the bedding of a cage on three successive days. Mice

were placed in these cages for three days. One group was fed in this contaminated cage, another in a separate clean cage, but there was no difference in the final results. All twelve mice were infected. Two died on the eighth and ninth days respectively, and the remainder were killed on the eleventh day. No primary lesions were found on three of the mice, including one of the fatal cases. All the others had primary lesions on the feet, belly wall or snout. In five there were two or more primary sores on the feet, belly and snout in various combinations. In the others only a single primary lesion was found.

Infection by Contact with Infected Mice.

Four mice which had survived pad inoculation with a 10–7 suspension of "Moscow" ectromelia virus had by the eleventh day developed considerable swelling of the inoculated feet with scabs. At two-day intervals, commencing eleven days after the inoculation, three normal mice were added to the four infected mice in a clean cage. At the end of each two-day period of exposure, the normal mice were removed to a clean tin and they were thereafter carefully examined each day. The results of this contact exposure are shown in Table 3.

TABLE 3.

Contact infection: normal mice added for two-day intervals to infected mice.

		Normal mice in contact.		
		Primary lesion.		Fate of animal.
Period of contact (from day of inoculation).	Condition of inoculated animals during this period.	Day of occurrence (after first exposure).	Site.	
Days 11 and 12	Inoculated feet swollen and scabbed (4)* conjunctivitis (2)	8	right groin	generalized skin rash followed by recovery
		9	left forefoot	generalized skin rash followed by recovery
		8	belly wall	†9**, acute ectromelia
Days 13 and 14	Inoculated foot healing (2) extensive generalized scabbing over body (2)	—	none found	†9, acute ectromelia
		7	side of nose	recovery
		7	right forefoot and nose	generalized skin rash followed by recovery
Days 15 and 16	Inoculated foot healed (2) extensive generalized scabbing over body (3)	5	belly wall	†10, acute ectromelia
		9	left groin	†11, acute ectromelia
		10	side of nose	†12, acute ectromelia
Days 17 and 18	Inoculated foot healed (3) extensive generalized scabbing over body (2)	—(A)	none found	†8, acute ectromelia
		14	lower lip	K18‡, acute visceral lesions. Probably secondary infection from (A)
		—	—	not infected
Days 19 and 20	Normal (2) extensive generalized scabbing over body (1) scab on nose (1)	9 (B)	side of nose	†14, acute ectromelia
		14	side of nose	†16, acute ectromelia, possibly secondary from (B)
		15	side of nose	K16, generalized skin rash, probably secondary infection from (B)
Days 21 and 22	Normal (3) healing generalized scabbing over body (1)	—	—	not infected
		—	—	not infected
		—	—	not infected
Days 25 to 31	Normal (4)	—	—	not infected
		—	—	not infected
			—	not infected

* (4) indicates that four etc. of the inoculated mice showed the lesions described.

**†9 = died nine days after first exposure to infected mice.

‡K18 = killed eighteen days after first exposure to infected mice.

The Appearance of the Primary Skin Lesion of Ectromelia.

If mice are examined daily after exposure to contact infection with ectromelia, a primary skin lesion can usually, but not always, be found in mice which become infected (Table 3). It is often detected better by touch than by sight, especially when it occurs in a thickly-furred part of the body. The first stage is a small brownish-coloured macule, but at this stage it is rarely found. When first noticed it is a small papule which increases in size fairly rapidly until it is about 4 mm. in diameter. The surrounding tissue is slightly inflamed and there is often considerable oedema of the subcutaneous tissue. Later the surface ulcerates and a scab soon forms. Healing occurs beneath the scab which usually drops off by the seventh day after the appearance of the papule. The primary lesion can easily be distinguished from the secondary skin eruption which often occurs in animals infected by a variety of routes which do not succumb to the acute infection. For instance, a generalized skin eruption occurred about the end of the second week in eleven out of fourteen mice which survived that time after infection by scarification of the foot with "Moscow" virus. This secondary eruption has been described by Marchal (1930) as crusts of dried serous discharge lying over superficially ulcerated areas. The time of appearance of the secondary rash, its widespread nature, and the absence of inflammation around each small scabbed area, distinguish these lesions from the primary sore.

Section of a primary lesion at the macular stage showed a localized area of necrosis of the skin and subcutaneous tissue. Pyknotic nuclei and nuclear fragments were scattered through the hyaline necrotic tissue. There were small areas of necrosis in the epithelium and subepithelial tissues surrounding the large necrotic area, but there was no cellular reaction. With Mann's stain the necrotic tissue was stained red and at the edges, where the staining was less intense, characteristic ectromelia inclusion bodies could be seen. In some sections of primary lesions at a stage slightly earlier than described above, there were very numerous inclusion bodies in the epithelial cells. The lymph node draining the primary lesion was usually enlarged and engorged and section of one such node showed massive necrosis.

Multiple Primary Lesions of Ectromelia.

When mice were exposed to infection in a cage, the sawdust bedding of which was very heavily contaminated with virus, several animals developed more than one lesion which was clinically diagnosed as a primary lesion. To determine the time relationships between the appearance of several primary lesions, virus was rubbed on the skin of mice with a small cotton-wool swab in several areas in the one day, and in other animals, in a different lightly scarified area each day for nine days.

When virus was applied to several areas at the same time, primary lesions appeared at all these areas together, appearing first as small macules on the fourth day, gradually becoming papular and later scabbed.

When virus was applied to several scarified areas on successive days, large lesions appeared in all animals four to five days after the local application of the virus in the areas treated on the first, second and third days. The lesions were smaller and had disappeared by the fifth day after scarification in the areas scarified on the fourth day. Subsequent to this, occasional positive results were obtained, one of three mice developing small lesions at the site of scarification of the tail on the eighth day. All animals were inoculated in the pad of the hind foot with 0·05 ml. of undiluted virus suspension on the thirteenth day after the beginning of the experiment. There was an accelerated response, with moderate swelling of the foot on the third day which had completely subsided by the fifth day.

DISCUSSION.

The experiments just described establish the fact that the skin is the usual portal of entry of ectromelia virus under natural conditions of transmission in laboratory mice. Like the viruses of warts, molluscum contagiosum, fowlpox, herpes, pseudorabies and lymphocytic choriomeningitis (Gordon, 1940) ectromelia can pass through the lightly scarified skin. It appears that it will not regularly pass through the completely intact normal skin, if suitable precautions are taken to allow minute abrasions to heal and to apply the virus with a minimum amount of trauma.

Bauer and Hudson (1928) reported that monkeys could sometimes be infected with yellow fever virus by rubbing it on to the intact normal skin. Positive results were obtained in only one of three trials and their experiments did not exclude trauma due to rubbing, and subsequent possible scratching of the site by

the monkey. Ectromelia virus always passed through the intact skin of the mouse when rubbed on with a cotton wool swab. Shaughnessy and Zichis (1940) investigating the passage of the virus of lymphocytic choriomeningitis through the skin of the young guinea-pig, sought to exclude trauma completely by carefully depositing the virus on the skin and covering the area with a protective capsule. They found that the virus of lymphocytic choriomeningitis would under these conditions pass through the intact skin. Neither of these writers records the development of a local lesion at the site of entry of the virus.

Mice which become infected with ectromelia by contact with infected mice or with a contaminated environment usually develop a local lesion at the site of entry of the virus. This primary lesion commences as a small macule, becomes papular, and later scabbed. Some animals, which die of acute ectromelia shortly after exposure, do not develop local lesion. A similar result, i.e. death without development of a local lesion of the foot, sometimes follows intradermal inoculation of the virus. In these animals the virus presumably enters the blood stream and causes death before sufficient reaction occurs at the site of entry to produce a visible lesion there.

The sites in which the primary lesion occurs are those parts which are most frequently in contact with the environment, i.e. the snout, the belly wall and the feet.

The other mammalian pox viruses which are related to ectromelia, namely smallpox, cowpox and rabbitpox, can infect their natural hosts through lesions of the skin of man, the cow and the rabbit respectively. This mode of infection was once thought to be the usual method of transmission of smallpox, but later investigations (Topley and Wilson, 1946) suggest that droplet infection via the nasopharynx is the principal method of infection. It appears certain that cowpox is usually transmitted from one animal to another by the milkers' hands, which may passively carry the virus or may have local cowpox lesions. In either case the virus enters the cow through small abrasions of the skin of the teats.

Rabbitpox is caused by a variant of neurovaccinia virus and is a highly contagious disease of rabbits (Greene, 1934; Pearce, Rosahn and Hu, 1936). Neither author discusses the route of entry of the virus in the natural infection, but Rosahn and Hu (1935) observe that it may be transmitted by a personal (human) carrier who handles susceptible animals after handling infected animals, even though the latter may not at the time show evidence of infection. The high frequency of nasal discharge and respiratory symptoms in the natural disease suggests that airborne transmission is probably also important. It is possible that natural infection may occur through the skin or through the nasal and conjunctival mucous membranes.

Cannibalism in a large mouse colony in which ectromelia was present might be expected to increase greatly the risks of infection of the mice. Animals which eat a mouse dying of ectromelia would probably become infected through abrasions of the snout or forefeet, and the dragging of heavily infected viscera on the bedding of the cage would greatly increase the chance of infection of other mice in the cage. In the experimental epidemics of ectromelia described by Greenwood *et al.* (1936), 11 p.c. of the mice which died could not be examined post-mortem because of cannibalism. Details of the times of occurrence of cannibalism in the ectromelia epidemics are not given, but in mouse typhoid and pasteurellosis epidemics it is stated that the extent of cannibalism varied rather widely from one secular interval to another, although not from one cage to another. Assuming that during the "steady state" period of the ectromelia epidemics the degree of infectivity of the environment to which the mice were exposed was constant, Kermack and McKendrick (1937) developed a mathematical theory of epidemics

which closely fitted the experimental observations. It is conceivable, however, that in closed epidemics, cannibalism would be most pronounced early in the epidemic, when the population is greatest and a large number are healthy. It might then exaggerate the high degree of infectivity which would be expected at this early stage of the epidemic.

SUMMARY.

Infectious ectromelia of mice is normally acquired through minute abrasions of the skin which come into contact with virus in the environment. Depending upon the natural resistance of the individual mouse, the virulence of the strain, and the dosage of virus, the infected mouse may die of acute ectromelia without developing a local lesion, or may develop a local lesion at the site of entry of the virus. Subsequently a mouse which develops a local lesion may die of acute ectromelia, develop a generalized cutaneous eruption and either die in the second or third week, or recover; or remain quite healthy, the local lesion healing by the end of the second week.

When successive separate infective doses enter the skin through abrasions in different areas, separate primary lesions usually occur when the first and last "inoculation" occur within five days and may occur when the interval is eight days.

In experimental epidemics of ectromelia cannibalism may be a major factor in contaminating the environment with virus and spreading infection.

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FURTHER STUDIES ON THE PHYSICAL STATES ASSUMED BY TOBACCO MOSAIC VIRUS *IN VITRO*[1]

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The physical state assumed by tobacco mosaic virus has been a subject of interest and often of controversy ever since Stanley (1935) crystallized the virus. As a result of X-ray diffraction patterns obtained with preparations of the virus, Bawden, Pirie, Bernal and Fankuchen (1936) classed the virus crystals as a new type of liquid crystal composed of aggregates having regular arrangement in two dimensions. The author described mesomorphic or paracrystalline fibres of the virus, first as spontaneous formations from clarified juice (Best, 1937a) and subsequently as artificially induced formations from pure virus solutions under the influence of electrolyte (Best, 1937b). Bawden considered it probable that these fibres were formed as a result of a combination of virus with some other plant constituent in spite of the author's claim to have produced them from pure virus. In the present paper it is shown that these long visible flexible paracrystalline fibres can be formed from the purest preparations of virus by suitably adjusting the concentrations of virus and electrolyte.

The separation of solutions of tobacco mosaic virus into two liquid phases, one of which is spontaneously birefringent and the other isotropic, first described by Bawden, Pirie, Bernal and Fankuchen (1936) were thought by Bawden and Pirie (1937) to be due to impurities in the solution and Bawden (1943) suggested that a perfectly pure solution of the virus would exist in one form or other but that the two would not co-exist in equilibrium. The author (Best, 1939) has demonstrated that the separation of solutions of tobacco mosaic virus into these two phases and the equilibrium concentration of virus are determined (at constant temperature and pH value) by the concentration of virus and electrolyte (NaCl) at low concentrations of the latter. Bernal and Fankuchen (1941) in a particularly thorough series of X-ray and crystallographic studies of the virus have, amongst other things, investigated this phenomenon in very concentrated salt-free virus solutions (above 13 p.c.) and recognize two distinct phases in equilibrium.

The results of observations and experiments described in the present paper go to show that the "needle crystals" of Stanley, the paracrystalline fibres of the author and the "layering" phenomenon appear to be stages in a regular sequence which, at constant temperature, is influenced by electrolyte concentration and the concentration of virus.

EXPERIMENTAL MATERIALS.

Source of Virus. Ordinary tobacco mosaic virus, prepared in a pure state by the standard method described by Best (1940a) from the juice extracted from plants of *Nicotiana Tabacum*, var. Blue Pryor, raised in a glasshouse and crushed about six weeks after they were artificially infected, was used in these investigations.

The virus contained 16·6 p.c. N and 0·5 p.c. P. The partial amino-acid composition was similar to that recorded by Best and Lugg (1944).

Reagents. Those of analytical grade or better were used throughout.

pH Value. Unless otherwise stated the pH value of all test systems was 7.

Temperature. All tests were carried out at 30° C.

[1]Glasshouse facilities and part of the cost of these investigations were supplied by the Council for Scientific and Industrial Research.

EXPERIMENTAL METHODS AND RESULTS.

At High Salt Concentrations (about 0·5 N and upwards).

The well known needle crystals or microtactoids formed at high salt concentrations have been described by numerous workers. The results of a study of their formation under controlled conditions and using a wide range of electrolytes have been recorded by Best (1940b). Photomicrographs published in that paper were of microtactoids precipitated from salt solutions at or above the critical coagulating concentration. When the concentration of electrolyte is just below the critical, a slower precipitation of virus from the thixotropic gel takes place. Mostly (but not always) the microtactoids formed are longer than those formed by more rapid precipitation. Fig. 1 is a photomicrograph of microtactoids formed under these conditions and is of interest because it shows what might be regarded as an intermediate stage between the tactoids formed by rapid precipitation and the long fibres described in the next section.

At Intermediate Salt Concentrations (univalent cations).

At concentrations of electrolyte still further below the critical value for rapid precipitation (24-hour limit) and down to about 0·2 N the tactoids which eventually settle out are long and fibrous. The length and strength of the fibres increase with time and they also vary with the nature of the anion. The behaviour and appearance of these fibres are shown in Figs. 2, 3.

Fig. 2a is a photograph of a glass tube containing a deposit of mosaic virus formed by slow precipitation from its aqueous solution by KBr. One ml. of 0·4 p.c. solution of pure virus in water was kept at 30° C. An equal volume of a solution of KBr was added to bring the concentration of the mixture to 0·64 M in respect to KBr. The concentration of virus in the final mixture was, therefore, of the same order as in the juice expressed from fully infected plants (Best, 1936). No precipitation of virus took place until well after twenty-four hours'

Fig. 1. Photomicrograph of birefringent microtactoids of tobacco mosaic virus formed by slow precipitation from a 0·2 p.c. solution of virus by potassium phthalate at pH 7 and 30° C. (×250).

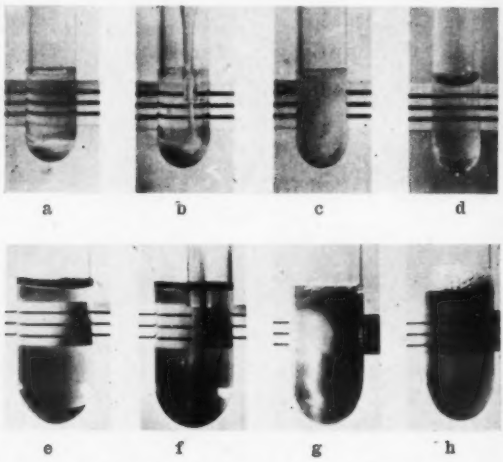
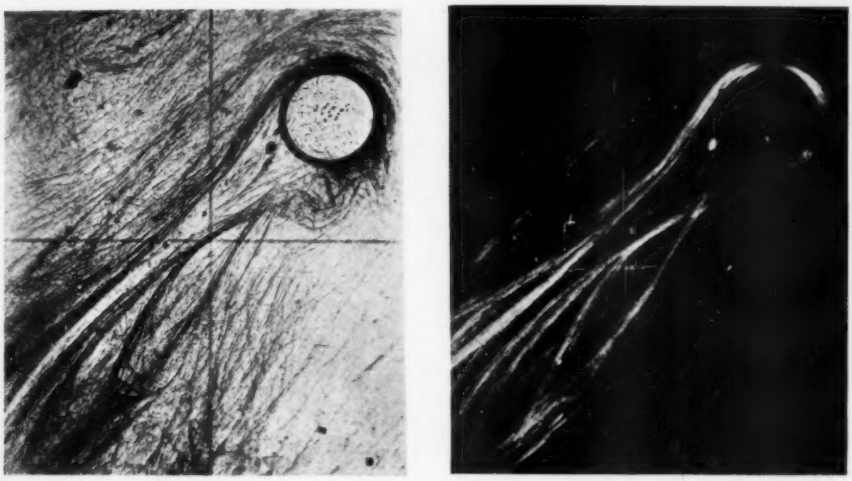


Fig. 2. a to d: Photographs of a tube containing fibres of tobacco mosaic virus formed by slow precipitation from a 0·2 p.c. aqueous solution of the pure virus by KBr at 0·64 M (pH 7, 30° C.); **a**: showing the white fibrous deposit at the bottom of the tube; **b**: a bundle of fibres being lifted from the deposit by a pipette; **c**: appearance after gentle agitation—an opaque suspension; **d**: appearance after vigorous shaking—a transparent sol.; **e** to **h**: photographs of a tube containing fibres of tobacco mosaic virus spontaneously precipitated from infective plant juice clarified by aeration and centrifugation at 2,000*g*. The various stages correspond with those of the tube above it.

contact. Such fibres normally form at concentrations of electrolyte further removed from the critical coagulating concentration than this, but the fibres here shown are quite typical.

This particular tube was one of a batch prepared in 1939 and put aside for observation. The photographs recorded in a–d were taken in 1940, sixteen months after the tube had been set up. Fig. 2b shows a bundle of fibres being lifted by a glass tube which had been dipped into the fibrous deposit and lifted after sealing the upper end with a finger.



a b

Fig. 3. Photomicrographs of long flexible paracrystalline fibres of tobacco virus formed by slow precipitation from a 0·2 p.c. solution of pure virus by 0·64 M KBr (pH 7, 30° C.). **a**: with ordinary light, **b**: between crossed nicols (×160).

Fig. 3 shows the appearance of a stationary field of the component fibres under the microscope by ordinary light and between crossed nicols. The bundle of fibres shown in Fig. 3 was caught up by a moving air bubble and took up a streamlined pattern, illustrating the flexibility of the fibres. There is a break on the lower arm of the bundle near the air bubble where a portion of the bubble broke through after the bundle had been brought under observation. Fig. 3b shows the spontaneous birefringence of the fibres and their straight extinction. These artificially formed fibres behave like those spontaneously formed ones described by the author previously (Best, 1937a). For example, photographs e–h of Fig. 2 correspond to the a–d series but are of a tube containing fibres spontaneously formed from clarified juice in 1936. The photomicrographs reproduced in the article in "Nature" (Best, 1937) and subsequently in Bawden's book (Bawden, 1943) were made from the fibre bundle shown in Fig. 2. The "naturally" and "artificially" formed fibres differed in that the latter were more easily broken up by mechanical forces. This is, very likely, due to the higher pH value at which they were formed (pH 7 as against a pH value between four and five for the clarified juice).

Fig. 2c shows the opaque suspension which was formed when the tube was gently agitated. The fibres exhibited a satin-like sheen as they were swirled round. Fig. 2d was taken immediately after giving the contents of the tube a vigorous shaking by hand. A clear transparent colloidal solution of the virus resulted. This is shown by the lines on the strip of cardboard placed behind the tube. The difference in the intensity of the background lines in a and d is mainly due to the opalescence of the virus solution (d) by reflected light. At this stage (d) the fibres had been broken up to sub-microscopic dimensions but on standing a few minutes the solution became cloudy and after an hour or so had reverted to the stage shown in c. After some days the stage shown in a and b was reached. This process appears to be reversible and can be repeated with the same tube at frequent intervals over a period of years.

However, the longer such a preparation is stored the faster does the re-aggregation process take place after dissolution, especially if the precipitating cation is a di-valent one. For example, a tube of the virus was set up in September, 1939, with $MgCl_2$ at just below its critical precipitating concentration. The tube was sealed and has been kept under observation

and shaken up at suitable intervals ever since. After seven years' storage the deposit of virus can still be made to spiral upwards as a fibrous bundle by rotating the tube suitably and can also be made to go through the stages c and d of Fig. 2 but the solution can no longer be reduced to the same degree of transparency by transmitted light as when it was freshly prepared. It appears that either the disaggregation brought about by vigorous shaking does not take place as readily with ageing or that the re-aggregation which takes place after the shaking is stopped is much speedier.

At Low Salt Concentrations.

Bawden, Pirie, Bernal and Fankuchen (1936) observed that solutions of tobacco mosaic virus in water separated into two liquid phases if the concentration of virus was above 2 p.c. The upper layer was isotropic when at rest but showed flow birefringence when disturbed. The lower layer was spontaneously birefringent and therefore liquid crystalline. This phenomenon has subsequently received a great deal of attention but little quantitative work has been recorded on the factors involved in the separation, except for that published by the author (Best, 1939) covering the effect of dilute salt solutions, and the comprehensive crystallographic studies by Bernal and Fankuchen (1941) of the more concentrated solutions of salt-free virus.

The author's earlier conclusions (Best, 1939) that the concentrations of electrolyte and of virus were the factors which determined whether or not the solution would separate into two phases and, if so, the relative proportions of each, is amplified by the data presented in Tables 1 and 2. The experimental procedure adopted to arrive at the data in these Tables was as follows:

In Table 1 are set out the equilibrium proportions (by volume) of the birefringent phase at various concentrations of virus and salt. 2 ml. of a solution of tobacco mosaic virus (birefringent bottom layer) containing 25 mg. virus per ml. (on a dry weight basis) were pipetted into each of a series of thin-walled 5 ml. pyrex tubes graduated in tenths of a millilitre. 0·5 ml. of water was added to the first tube and 0·5 ml. of NaCl solution to each of the seven others. The concentrations of the NaCl were chosen so that the final concentration of salt after mixing would correspond to the following molarities: 0·0005, 0·001, 0·002, 0·003, 0·004 and 0·007. The concentration of virus in each of the tubes was thus 20 mg. per ml. at this stage. The tubes were placed into a water bath controlled at 30° C. The contents were shaken by hand at intervals and allowed to settle. The volumes occupied by the two phases were read and the contents of each tube were again shaken. This process was repeated until the constancy of the volumes indicated that equilibrium had been established. Each tube was then diluted with a solution of NaCl of the appropriate concentration and the shaking and settling procedures were repeated. The progressive dilutions produced a range of virus concentrations from 20 to 8 mg. per ml., as shown in Table 1.

TABLE 1.

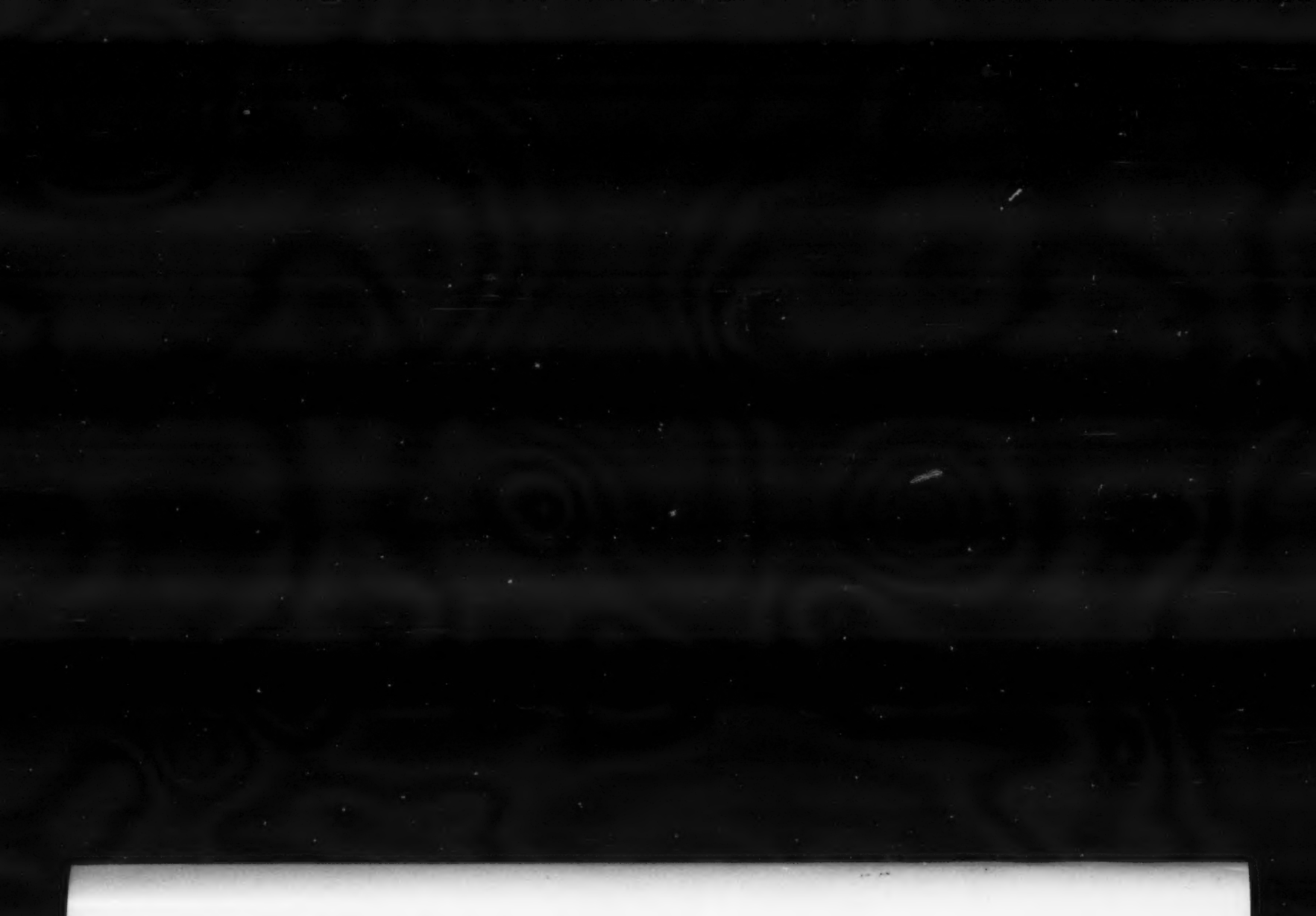
Showing the equilibrium proportion (by volume) of the birefringent phase of tobacco mosaic virus at various concentrations of virus and sodium chloride (30° C., pH 7).

Concentration of virus.	Molarity of sodium chloride.							
(mg./ml.).	0*	·0005	·001	·002	·003	·004	·005	·007
	Percentage birefringent phase (by volume).							
20	83	85	72	50	33	25	20	0
17·5	—	67	50	30	12	0	0	
15	48·5	44	24	12	0			
12·5	31	23	1	0				
10	8	0	0					
9	3							
8	0							

* Additional values in the absence of salt are 77 p.c. and 55 p.c. birefringent phase at virus concentrations of 18 and 16 mg./ml. respectively.

In Table 2 the distribution of the virus (by weight) between the two phases at various concentrations of sodium chloride is set out.

Reaction mixtures were prepared from pure virus by adding water or a solution of NaCl to give tubes in which the concentration of NaCl was 0, 0·001 M, 0·002 M, 0·0004 M and 0·005 M respectively. The concentration of virus was 20 mg. per ml. in each. However, the control tube and the 0·001 M salt tube gave so little of the isotropic layer that the concentration of virus in the former was brought to 15 mg. per ml. by adding water and that in the other was reduced to 17·5 mg. per ml. by adding 0·001 M NaCl in order to get enough of the isotropic



phase for analysis. The tubes were shaken at intervals of about 12 hours and the contents were allowed to settle. This process was continued until the relative volumes of the phases remained unaltered after two successive shakings. The time taken for the mixtures to settle so as to give a sharp interface varied with the salt concentration, as also did the length of time taken to reach the criterion chosen for the equilibrium state—namely constancy of the relative volumes of the two phases. At first the volume of the birefringent layer got smaller after each shaking but the changes were smaller after each shaking and eventually no further change took place. The 0·005 M NaCl system had reached equilibrium in 24 hours (2 shakings) whereas the salt-free system took eleven days. Because of the possibility of other changes taking place (ageing for example) the tubes were removed and analysed after eleven days. Aliquots were withdrawn from the top and bottom liquids in each tube and the virus content was determined by evaporation to dryness, storage over P_2O_5 and weighing. For the system containing salt a correction was made by assuming that the NaCl was evenly distributed throughout the system.

TABLE 2.

Showing the concentration of tobacco mosaic virus in the isotropic and birefringent phases in equilibrium at various concentrations of sodium chloride (30° C., pH 7).

Molarity of NaCl.	Concentration of virus (mg./ml.). Isotropic layer.	Birefringent layer.	Ratio Isotropic birefringent.
0	14·4	23·2	0·62
0·001	16·9	24·1	0·70
0·002	18·9	27·0	0·70
0·004	19·8	29·3	0·68
0·005	20·0	29·6	0·68

DISCUSSION.

Progressive dilution of the test system recorded in Table 1 caused a progressive reduction in the overall concentration of virus. It will be seen that the lower the overall concentration of virus the lower was the proportion of birefringent phase until at some limiting value of virus concentration only the isotropic phase was present. In the absence of added salts the birefringent phase persisted on dilution until a limiting overall virus concentration of about 0·9 p.c. was reached. This limiting concentration increased rapidly with increasing salt concentration until at 7 millimols of NaCl per litre no birefringent phase could exist at an overall virus concentration of 2 p.c.

These limiting concentrations are very much lower than those recorded by Bawden and Pirie from sporadic observations. These workers emphasized that the separation into phases takes place only after a certain degree of purity has been reached and that the purer the virus preparation the lower the virus concentration at which the two phases can co-exist. Their preparations usually began to separate at about 4 p.c. and for their purest preparations they named a limiting concentration of 1·8 p.c. In the author's experience preparations separate into phases very early in the purification process (often after two precipitations) and, as the data of Tables 1 and 2 show, the limiting virus concentrations for the co-existence of the two phases is about half that found by Bawden and Pirie. It would therefore appear that the author's preparations are very pure.

If we plot the percentage of birefringent phase (by volume) against the overall concentration of virus at each salt concentration we get a series of straight lines. Taking them as a group these are parallel within the experimental error except for the 0·0001 M NaCl system which has a steeper slope. In view of the general approach to parallelism the departures from this may be due to incomplete equilibrium in some cases. It is difficult to decide when a colloidal system such as this has reached true equilibrium. In any case, as they cannot be far out, a useful purpose is served by extending the lines beyond the experimental range and extrapolating to get the limiting overall concentrations of virus for the range

over which the two phases can co-exist. When this is done, we find that the respective ranges for NaCl concentrations of 0, 0·5, 1, 2 and 3 millimols per litre are 0·9 to 2·1, 1·0 to 2·2, 1·25 to 2·3 or 2·4, 1·35 to 2·7 and 1·6 to 2·85 p.c. virus, and if we draw parallel lines through the single points available for 4 and 5 millimols of NaCl, we get corresponding ranges of 1·7 to 2·95 and 1·8 to 3·0 p.c. virus. Outside these limits we would expect to get only one phase (i.e. when the overall virus concentration is 2 p.c.). These figures agree quite well with actual determinations recorded in Table 2.

From the data of Table 2 it is seen that as the concentration of NaCl increases the concentration of virus in both phases also increases. The volume of the more concentrated birefringent phase gets progressively smaller as the salt concentration increases. This is best observed from the last three lines of Table 2 where the total volume of the system and overall virus concentration is the same for all three salt concentrations. The ratio of the concentrations of virus in the two phases appears to be reasonably constant except for the salt-free system. However, the changes in virus concentration of any one phase at different salt concentrations are so small that since both concentrations are increasing the constancy of the ratio may be fortuitous. A larger series of determinations over a wider range of virus-salt concentrations will be needed to test the significance of this apparent constancy. If the constancy is found to be real or if some modified form of the ratio is established as a constant the theory behind these phase separations will be helped considerably. But whether the constancy of the ratio has any theoretical significance or not it is a constant within the experimental error over the range investigated and as such can be used to calculate other values within the range studied. The value given in Table 2 for the systems containing 2, 4 and 5 millimols of NaCl per litre refer to the same overall virus concentration of 2 p.c. For reasons stated earlier the systems containing 0 and 1 millimols NaCl per litre contained overall virus concentrations of 1·50 and 1·75 p.c. respectively. Assuming that the ratio of 0·7 for the distribution of virus between the two phases (expressed as concentrations) would hold also for these two systems if the overall virus concentrations in them had been 2 p.c., we can calculate values for the weight of virus in each phase and so bring them into line with the other three systems. If these two re-calculated values and the other three experimentally determined values for the weight of virus in the birefringent phase are plotted against the concentration of NaCl all five points fall on a straight line with a slope corresponding to 2·5 mg. virus per millimol NaCl. This line cuts the salt axis at 0·007 M NaCl. That is at 0·007 M NaCl there should be no birefringent phase when the overall virus concentration is 2 p.c. This value derived from the data of the experiment summarized in Table 2 agrees very well indeed with the observed value of the experiment summarized in Table 1. To get the two phases to co-exist in equilibrium at and above a concentration of NaCl of 0·007 M it will, therefore, be necessary to have a solution of virus more concentrated than 2 p.c.

To explain the various phenomena Bernal and Fankuchen (1941) have developed a theory based on an extension of Levine's (1939) theories of the interaction of charged colloidal particles. They have shown that the inter-particle distance of the virus in the sols decreases with increasing virus concentration and also decreases with increasing salt concentration for solutions of ammonium sulphate more concentrated than about 0·3 N. They suggest that the length of the virus particle may be the variable which determines the limiting concentrations of virus in the two-phase system (they also allow that "the effect of unidentified impurities may be of as great or greater importance").

According to their theory as the length of the particles increases both equilibrium concentrations should decrease. Since the addition of salt also has the effect of decreasing the length of the particles the virus concentration of both phases should increase with increasing salt concentration. This is in fact what is observed in the data of Table 2, which therefore give qualitative support to their theory. Bernal and Fankuchen themselves did not work with solutions less concentrated than 13 p.c. virus and the only figures given under the heading of "dilute salt solutions" refer to concentrations of $(NH_4)_2SO_4$ of 0·1 N to 0·3 N. These concentrations are at least fifty times greater than those referred to as "dilute" in the present paper, so that their dilute solutions come within the range of intermediate salt concentrations according to the author's terminology.

Bernal and Fankuchen also report anomalous behaviour when dilute salt solutions are added to an equilibrium mixture of the two phases. In their experiments the birefringent phase increased in volume and they referred to this as " paradoxical" behaviour. Their experiments on this point were rough and, unfortunately, they did not report the actual salt concentrations concerned. However, it seems likely that they were in the intermediate range (0·1 to 0·3 N) and, therefore, not far below another critical condition, viz. the critical salt concentration for rapid coagulation. At the low salt concentrations used in the present work the volume of birefringent phase decreased as the salt concentration increased. This also fits in with their theory.

Paracrystalline Fibres.

These are formed at intermediate salt concentrations, below the critical coagulating concentration (for microtactoids) and above those discussed in the previous section. That the virus preparations from which the fibres shown in Fig. 3 were formed were particularly pure, as mentioned earlier in this paper, is further borne out by their general behaviour and analyses (see e.g. Best and Lugg, 1941). Bawden's suggestion that the fibres described by the author are likely to be complexes between virus and other constituents of the cell sap cannot be supported. On the contrary, the view expressed by Bawden and others that the presence of impurities prevents the aggregation of the virus particles into larger units and that this aggregation takes place more readily the purer the preparations supports the author's conclusions. The conclusion arrived at by Bernal and Fankuchen (1941) that the fibres described by the author (1937) are an extreme form of tactoid would appear to be more reasonable.

Microtactoids.

At concentrations of electrolytes above the critical for rapid coagulation, the virus separates out as microtactoids the size of which depends, amongst other things, on the concentration of electrolyte. The tactoids shown in Fig. 1 illustrate an intermediate stage or form between the long fibre and the short microtactoid. A close examination of the photograph (under magnification) will reveal that the virus seems to have formed into shorter fibres or longer rods by the end to end joining up of a number of microtactoids which fused at their points of contact. The shorter units appear to have joined together with a slight overlap and to have fused at the point of contact thus giving the aggregate the appearance of a spiral or drill. There are also in the field some strands of what appear to be aggregates formed by two or more such long threads joining laterally and giving the appearance of a plait.

SUMMARY.

Data concerning the physical states assumed by pure preparations of tobacco mosaic virus at various concentrations of virus and NaCl are presented and discussed.

In the absence of salt we get isotropic sols if the concentration of virus is less than about 1 p.c., a two-phase system of isotropic sol and birefringent crystalline liquid in equilibrium if the overall concentration of virus is above 1 p.c. and below about 2 p.c., and only birefringent liquid phase at higher virus concentrations.

At low salt concentration ($<$ ·01 M for univalent salts) the sequence is the same but the limiting concentrations of virus are higher. The transition from isotropic sol only to the two-phase system occurs at a higher virus concentration the higher the salt concentration. Within this system the concentration of virus is higher in the birefringent phase than in the isotropic phase and the concentration of virus increases in both phases with increasing salt concentration. The volume of birefringent phase decreases with increasing salt concentration.

The lower limit of virus concentration below which only isotropic sol exists and above which the two liquid phases co-exist was found by successive dilution to be 0·9, 1·2, 1·3, 1·7, 1·8, and 2·0 p.c. at salt concentrations of 0, 1, 2, 4, 5 and 7 millimols of NaCl per litre respectively.

In the two-phase system with an overall virus concentration of 2 p.c. the equilibrium concentrations of virus (expressed as p.c. dry virus) at salt concentrations of 0, 1, 2, 4 and 5 millimols NaCl per litre were found to be 1·44, 1·69, 1·89, 1·98 and 2·00 p.c. respectively in the upper isotropic phase and 2·32, 2·41, 2·70, 2·93 and 2·96 p.c. respectively in the lower birefringent phase.

At intermediate salt concentrations, the upper limit varying with the nature of the salt roughly between 0·1 N and 1·0 N, the virus is precipitated slowly in the form of birefringent tactoids. These assume the form either of long flexible fibres or of shorter aggregates as shown in the accompanying photomicrographs.

At high salt concentrations (at and above the coagulating concentration for rapid precipitation of virus, i.e. from about 0·5 N and upwards) the virus is precipitated from solution in the form of birefringent microtactoids.

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ISCHAEMIA, ANOXAEMIA AND SHOCK

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Nathanson, Nutt, Pope, Zamecnik, Aub, Brues and Kety (1945) developed a technique allowing the collection of fluid from a muscle after ischaemia was produced by the application of a tourniquet for some hours. If the fluid was not collected shock developed after release of the tourniquet in three out of ten cases. Muscle material is absorbed during this period and there is an exudation of fluid (Nathanson *et al.*, 1945). No significant changes in the extra-cellular space of the body of the animal developed except in the area of local trauma. Sodium and chloride levels in the plasma were unaltered but there was a terminal increase in the plasma potassium. The muscle gained sodium and lost potassium (Brues *et al.*, 1945). There was a loss of fluid into the leg locally from the body (Kety *et al.*, 1945). The exudate which accumulated and was collected produced shock in one animal in four following injection (Aub *et al.*, 1945). An effort was made to isolate the toxic factor present in the exudate and it was indicated that changes in electrolyte were insufficient to cause shock. Dialysis and fractionation indicated that the protein fraction contained the toxic substance. Muscle enzymes were suspect but proteolytic enzymes examined were found not be toxic (Zamecnik *et al.*, 1945). Bacterial infection was thought to play a part (Pope *et al.*, 1945).

In this investigation muscle fluid was obtained following ischaemia in the intact dog and the fluid so obtained was investigated for the presence of histamine, slow-reacting substance (S.R.S.), cardio-depressant substance and deaminating enzyme. Histamine and S.R.S. were found not to be liberated. Traces of cardio-depressant substance were found in the centrifuged fluid and considerable amounts from the whole fluid when boiled. Very large amounts of deaminating enzyme were detected in the centrifuged fluid.

Also in this investigation the liver has been perfused with diluted blood from the same animal and the liberation of deaminating enzyme has been examined. It was found that by slowing the rate of flow increased amounts of this enzyme were liberated and this was further related to the oxygen supply of the liver tissue: it was found that the enzyme appeared in increased amounts as the oxygen supply was reduced.

METHOD.

Muscle Experiment.

Under Pentobarbital anaesthesia (45–65 mg. per kg. i.v. in divided doses) the left triceps-surae muscle of the dog was prepared as described by Nathanson *et al.* (1945), the tourniquet left on the muscle for 3½–5½ hours and fluid collected from the sheath into a chilled flask lying in ice water for 1½–4½ hours. In some cases (Nos. 3, 4, 5, 7 and 16) saline (5 p.c. v/w animal) was introduced into the external malleolar vein. The saline was begun 10–15 minutes before the tourniquet was released, and was run in for 1½–3 hours. The fluid so obtained was centrifuged and sediment was tested for cardio-depressant activity after taking up in Tyrode and boiling. The supernatant fluid was similarly tested after boiling. This fluid was also tested for deaminating enzyme activity prior to the boiling by mixing it with adenosine (1/19, 1/9 or 1/4 of its volume of 1:1,000 adenosine in Tyrode) and incubating it for 30 minutes at 39° C. while 4·65 p.c. CO_2 in oxygen was passed over its surface after bubbling through water. Plasma from heparinized blood obtained from the dog at the end of the experiment was similarly treated, thus maintaining constancy of pH (Trethewie, 1942b). Similarly uncentrifuged fluid was tested for deaminating enzyme activity. The cardio-depressant activity of the system was

measured on the auricle of the guinea-pig as described by Drury, Lutwak-Mann and Solandt (1938). The reduction in cardio-depressant activity after incubation for 30 minutes when adenosine was added was taken as an index of the enzyme content of the mixture.

Liver Perfusion.

Cats were anaesthetized with Nembutal (75–95 mg./kg. intraperitoneally). Dogs were anaesthetized with Pentobarbital (36 mg./kg. i.v.). 50 ml. of blood were run from the aorta of cats into heparin in a cylinder and diluted in 450 ml. of Tyrode. 150 ml. of blood were run from the aorta of dogs into heparin in a cylinder and diluted in 1,350 ml. of Tyrode. The operation was performed aseptically and the liver perfused with Tyrode through the portal vein and placed on a paraffin block as described by Feldberg and Kellaway (1937b); then the diluted heparinized blood (0·012 mg. heparin, Boots, per ml.) was used for perfusion, the rate of flow being 1–7 ml. per minute, and temperature 37° C. The diluted blood used for perfusion was agitated regularly. Perfused fluid (P) was collected as perfusate and centrifuged at 4,000 r.p.m. for 30 minutes and diluted plasma so obtained. Control fluid (C) was obtained from the flask delivering the diluted blood and similarly centrifuged and plasma obtained. Tests for deaminating enzyme activity were made as before for undiluted dog plasma.

Nitrogen Exposure.

In these investigations nitrogen (containing 1 p.c. O_2 and less than 0·1 p.c. CO_2) was bubbled through water before exposure over a large flask containing the diluted blood and the nitrogen exposure was continued throughout the experiment. With controls the flask was placed in a bath at 37° C., except in one instance.

The oxygen capacity of the diluted blood was carried out in a Van Slyke blood gas analysis apparatus by Miss Joan B. Cleland, who also estimated the purity of the nitrogen.

Histamine and S.R.S. (Slow-reacting Substance).

Histamine and S.R.S. were assayed on the guinea-pig jejunum suspended in a bath of Tyrode (Feldberg and Kellaway, 1938b). The samples were left in contact with the bath for 30 seconds. Control histamine was histamine-acid phosphate.

Muscle Fluid Bacteria.

Samples were plated out on blood plates and inoculated into meat media and serum broth. The identification of the organisms was carried out by Miss Nancy Atkinson.

EXPERIMENTAL.

MUSCLE EXPERIMENT.

Deaminating Enzyme Activity of Fluid Obtained from Ischaemic Muscle.

In six experiments the fluid passing out of the muscle was collected and centrifuged. The supernatant fluid was designated "fluid" (Table 1) and heparinized plasma was obtained from the external malleolar vein at the conclusion of the experiment and designated "plasma" (Table 1). Adenosine was made up (1:1,000 or 1:2,000) and added to the fluid to give a concentration of 1:25,000 and a similar amount added to the plasma. When this is done the cardio-depressant activity of the solution when boiled just after mixing is not always equal to that of a similar mixture of adenosine and Tyrode, e.g. in one experiment the muscle fluid was mixed with adenosine (1:25,000) and a portion boiled prior to incubation showed an activity of 75 p.c. (= adenosine 1:33,000): this phenomenon has been found elsewhere, e.g. in pneumonia (Trethewie, 1945a). Following incubation for 30 minutes at 39° C. it was found in every instance (No. 1–5, Table 1) that the system containing the fluid was completely inactivated. In one, experiment 1, incubation was for 40 minutes. On the other hand the plasma only reduced the activity of the system to 50–70 p.c. in the same time. This corresponds with the activity of normal human plasma (Trethewie, 1942b). In experiment 5 adenosine was then added in a concentration of 1:10,000 to the fluid and incubated for 30 minutes. Complete inactivation again occurred. In experiment 6, the system was made up to 1:5,000 and the fluid, after 30 minutes' incubation, showed an activity of 12·5 p.c. and the plasma 82 p.c. These findings are indicated in Fig. 1. At C a large response was produced by 0·5 ml. of plasma after incubation with adenosine 1:5,000 and diluted 1/5. A is the response to 0·5 ml. adenosine 1:100,000 (5γ). At F a small response is produced by 0·6 ml. of the fluid after incubation with adenosine 1:5,000 and diluted 1/5. Thus there is a very considerable increase in deaminating enzyme activity of the centrifuged supernatant fluid as compared with plasma and therefore the activity of the fluid cannot be held due to simple outpouring of plasma. Further, the fluid on standing shows a fall in pH. A slight rise in pH causes a slight increase in deaminating enzyme activity (Trethewie, 1942b) while a large fall or rise causes an inhibition of enzyme activity (Drury, Lutwak-Mann and Solandt, 1938). Therefore, changes in pH cannot account for the increased enzyme activity of muscle fluid.

The findings in all these cases are shown in Table 1.

TABLE 1.

Deaminating enzyme activity of muscle fluid compared with animal's own plasma.

Dog No.	Fluid vol. ml.	Conc. system adenosine.	p.c. activity after 30 mins'. incubation. fluid.	plasma.
1.	30	1: 25,000	0	70*
2.	20	1: 25,000	0	50
3.	500	1: 25,000	0	65
4.	16	1: 25,000	0	65
5.	60	1: 25,000	0	60
		1: 10,000	0	
6.	5	1: 5,000	12·5	82

* Incubated 40 minutes

The details of one experiment were as follows: The muscle of a dog (2) weighing 6·8 kg. was prepared and the tourniquet left on for 4½ hours. At four hours 340 ml. saline were run slowly into a vein for three hours. Twenty ml. of fluid were collected for 4½ hours after releasing the tourniquet. The fluid was centrifuged and the supernatant when mixed with adenosine in a concentration of 1:25,000 contained no activity after incubation at 39° C. for 30 minutes. Heparinized plasma obtained from the vein when mixed with adenosine in the same concentration and incubated for 30 minutes at 39° C. had an activity equal to 50 p.c. These findings are shown in Fig. 2. At A is shown the response to 0·5 ml. adenosine (1:125,000). At B no response is shown to 1 ml. of centrifuged fluid after incubation with adenosine (boiled and diluted 1/5) and at C a response equal to 0·4 ml. adenosine (1:125,000) (A_1) is shown by 0·8 ml. of plasma after incubation with adenosine (boiled and diluted 1/5).

Fig. 1.

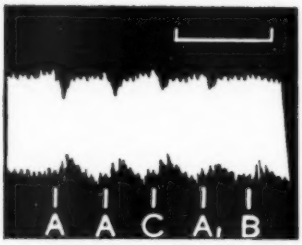

Fig. 2.

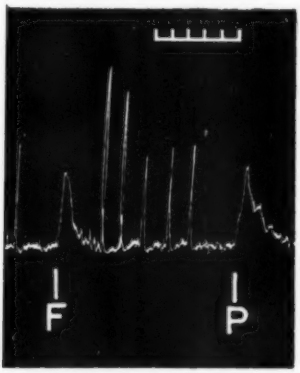

Fig. 3.

Fig. 1. Responses of the guinea-pig auricle to samples from dog 6 diluted 1/5. At C response to 0·5 ml. of plasma after incubation with adenosine. At F response to 0·6 ml. of muscle fluid after incubation with adenosine. At A response to 0·5 ml. adenosine (1:125,000). Time in minutes. Details in text.

Fig. 2. Responses of the auricle of the intact guinea-pig. Samples tested from dog 2. At A and A′ responses to 0·5 ml. and 0·4 ml. adenosine (1:125,000). At C response to 0·8 ml. of plasma after incubation with adenosine and diluted 1/5. At B, 1 ml. of muscle fluid after incubation with adenosine was tested (diluted 1/5). Time in minutes. Details in text.

Fig. 3. Response of the isolated guinea-pig jejunum to muscle fluid (boiled) and plasma (boiled) obtained from a dog weighing 7·2 kg. At F the response to 0·4 ml. muscle fluid, latency three seconds. At P response to 0·4 ml. plasma, latency six seconds. The unlettered responses are to 0·05γ histamine. Time in minutes.

In experiment 3 the uncentrifuged fluid was also tested for deaminating enzyme activity. After incubation for 30 minutes at 39° C. boiling and diluting it was found that the activity was 90 p.c. that of the added adenosine. This apparent poor enzyme activity is due to the fact

that the cellular material has considerable cardio-depressant activity. In another experiment (7) 3 ml. of the fluid collected during 45 minutes was removed from the chilled flask, centrifuged and the sediment taken up in 2 ml. of Tyrode and boiled. 0·7 ml. of this showed considerable cardio-depressant activity which was greater than that of 5γ adenosine. Thus when adenosine is mixed with whole fluid and incubated there is an already present degree of cardio-depressant activity which militates against an orderly assessment. Further, enzymes themselves may be liberated from the cellular material during incubation and prior to boiling.

The centrifuged muscle fluid was found to be haemorrhagic and some haemolysis had occurred. Some of the colour was probably due to muscle protein. Since haemolysis increases deaminating enzyme activity (Trethewie, 1946) it was decided to test the effect of haemolysis of the degree found to determine if its effect were significant. In one experiment (3) where the muscle fluid gave a Hb. value of 0·07 gm. p.c. the fluid completely inactivated 1:25,000 adenosine at 39° C. after 30 minutes' incubation. Haemolysed blood when mixed with adenosine and plasma (which alone reduced the activity of the adenosine to 65 p.c.) increased the activity to reduce the adenosine to 20 p.c. after incubation, while the Hb. estimation of the system was 1·3 gm. p.c. Thus, in the degree found, haemolysis was insignificant in its effect on deaminating enzyme activity. The muscle fluid whether markedly haemolysed or not always completely inactivated a solution of 1:25,000 adenosine.

The Liberation of Cardio-depressant Substance into the Ischaemic Fluid.

It has already been shown that the uncentrifuged fluid on boiling has considerable cardio-depressant activity; but this is an artificial procedure. It is more physiological if the fluid as it passes out of the muscle is tested immediately for cardio-depressant activity and this has been done. It is then necessary to test centrifuged fluid. It was pointed out by Green (1943) that some of the shock produced in muscle ischaemia might be due to the liberation of adenosine-triphosphate but evidence for this was lacking and Macfarlane and Spooner (1946) summed up the evidence which was not in favour of compounds of this type producing shock. However, occasionally in shock a bradycardia is produced and it is not certain that in the few instances where this occurs such an effect is not produced by the liberation of a substance of this type (adenyl compound). In tests performed on the centrifuged fluid obtained immediately from the chilled flask in one instance no cardio-depressant activity was detected. In this experiment 0·45 ml. of the supernatant fluid (diluted 1/2) was without effect on a heart which responded to 2γ adenosine. In another experiment supernatant fluid obtained from the chilled flask 45 minutes after the tourniquet was released and boiled had cardio-depressant activity equal to approximately 0·5γ adenosine when tested on a sensitive preparation.

Absence of Liberation of Histamine and Slow-Reacting Substance (S.R.S.) in Fluid Obtained from Ischaemic Muscle.

The supernatant muscle fluid was tested for its response on the isolated guinea-pig jejunum and an attempt was then made to determine if it contained either histamine or S.R.S. (Feldberg and Kellaway, 1938b; Kellaway and Trethewie, 1939b). No histamine was detected on assay and no increase in S.R.S. was found over that contained in plasma. In one experiment (4) 16 ml. fluid were obtained from a dog weighing 7·2 kg. and the fluid centrifuged immediately. Portion of the supernatant was boiled and when 0·4 ml. was injected into the bath the gut did not respond till three seconds had elapsed. A rapid response followed and the bath was washed out at 30 seconds. This is a typical S.R.S. response (Fig. 3, F) and it was very closely equalled by that to 0·4 ml. of plasma (P) obtained from the same dog and boiled immediately where the response occurred after an interval of six seconds. The response to histamine is an immediate one and thus there is no evidence that histamine is liberated in any considerable concentration. There is also no evidence that S.R.S. appears in the muscle fluid in any higher concentration than that already present in the plasma.

Bacterial Content of Fluid.

Examination of the muscle fluid for organisms in four cases revealed *Cl. welchii* once and staphylococcus (non-haemolytic, coagulase negative) four times (Table 2).

TABLE 2.

Organisms in muscle fluid.

Exp. No.	Organism.
1.	*Cl. welchii* Staphylococcus (non-haemolytic, coagulase negative).
4.	Staphylococcus (non-haemolytic, coagulase negative).
6.	Staphylococcus (non-haemolytic, coagulase negative).
7.	Staphylococcus (non-haemolytic, coagulase negative).

LIVER PERFUSION.

The Liberation of Deaminating Enzyme from the Isolated Liver Perfused with Diluted Blood.

Cat. When the liver of the cat was perfused with diluted blood at a rate of 1–7 ml. per minute the deaminating enzyme activity of the fluid after perfusion was greater than that of the control unperfused fluid. Normal plasma contains deaminating enzyme as has been shown by Drury, Lutwak-Mann and Solandt (1938).

In one experiment (8) the liver weighing 141 gm. was perfused at a rate of 3·3 ml. per minute. The control unperfused plasma when removed after centrifugation and mixed with adenosine 1:20,000 reduced the activity to 80 p.c. after 30 minutes' incubation at 39° C. The perfused fluid, 100 ml. collected during 30 minutes' perfusion after centrifugation, left plasma which inactivated 60 p.c. of the 1:20,000 adenosine system, leaving only 40 p.c. activity after 30 minutes' incubation. In another experiment (14) where the rate of flow was 7 ml. per minute and the liver weighed 105 gm. the perfused fluid after centrifugation reduced the activity of 1:20,000 adenosine to 33 p.c. Details of all the cat liver perfusions are shown in Table 3.

TABLE 3.

Deaminating enzyme activity of control and perfused fluids from cat experiments.

No.	Vol. ml. perfused fluid.	Time mins.	Conc. system adenosine.	p.c. activity after 30 mins'. incubation	
				Control fluid.	Perfused fluid.
8.	100	30	1:20,000	82	40
	20	20			0
9	—	—	1:20,000	100	
				N_2 97	
10.	50	25	1:20,000	40	0
	N_2 50	25		40	0
11.	100	22	1:20,000	30	20
	N_2 50	11		25	0
12	112	15	1:20,000		18
	N_2 40	6			0
13	85	12	1:20,000		43
	N_2 44	6			30
14	70	10	1:20,000		33
	100	14			35

Dog. In a dog (15) perfusion where a portion of liver weighing 180 gm. was perfused at a rate of 3·9 ml. per minute the diluted plasma obtained after perfusion reduced the activity of 1:20,000 adenosine after 30 minutes' incubation to 90 p.c. The control unperfused diluted plasma was without effect when mixed with adenosine 1:20,000 and incubated for 30 minutes.

The Effect of Rate of Flow on the Liberation of Deaminating Enzyme.

Now, in these experiments the flow was less than normal and further the fluid was not whole blood but diluted. It was thought that it might be owing to these abnormalities that the deaminating enzyme activity of the fluid was greater after perfusion. The rate of flow was then reduced and we were able to compare the activity of perfused fluid from the same liver when the rate of flow was varied with that of unperfused diluted plasma. These findings are shown in Table 4.

TABLE 4.

Effect of rate of flow on enzyme activity.

	No.	Perfusion rate ml./min.	Wt. liver gm.	Conc. adenosine.	Activity after 30 mins'. incubation.
Dog	15.	Control	180	1:20,000	100
		3·9			90
		0·7			35
Cat	8.	Control	141	1:20,000	80
		3·3			40
		1			0
Cat	14	7	105	1:20,000	33
		7·1			35

Cat. In experiment 8 (cat) the rate of flow was reduced from 3·3 ml. per minute when the perfused diluted plasma had an activity reducing the concentration of the adenosine system to 40 p.c. after incubation, to 1 ml. per minute for 20 minutes and the perfused fluid now completely inactivated the system of 1:20,000 adenosine in 30 minutes.

As a control (No. 14, cat) the rate of flow was continued at the same rate for the collection of the samples and the sample collected at 7 ml. per minute for 10 minutes reduced the activity of the adenosine system to 33 p.c. after 30 minutes' incubation and the sample collected for the next 14 minutes (100 ml.) produced diluted plasma which inactivated the same system to 35 p.c. activity in the same time.

Dog. In experiment 15 (dog) the rate of flow was reduced from 3·9 ml. per minute (75 minutes) (when perfused plasma reduced the activity of the adenosine system to 90 p.c.) to 0·7 ml. per minute for 135 minutes and the diluted plasma after perfusion then had increased in activity to reduce the activity of the adenosine system to 35 p.c. after incubation. The control diluted plasma was without effect on this system after 30 minutes' incubation. These findings are indicated in Fig. 4. A is the response to 10γ adenosine, A′ to 4γ and A″ to 8γ adenosine. The response at B to the sample of plasma after incubation prior to perfusion is equal to 10γ adenosine. At C the response to plasma obtained at the perfusion rate of 0·7 ml. per minute is equal that of 4γ adenosine. The response at D to plasma after incubation collected at the perfusion rate of 3·9 ml. per minute is intermediate in activity between 10 and 8γ adenosine. Responses to samples of plasma are all to 1 ml.

Therefore, when the rate of flow through the organ is reduced there is an increased output of deaminating enzyme into the diluted blood. This effect might be due to a diminished supply of oxygen to the organ and in fact the same explanation may be true of the output of deaminating enzyme even with the less severely slowed rate of flow.

The Effect of Replacing the Air Above the Diluted Blood Flask Delivering the Perfusion Fluid with Nitrogen (Cat).

Blood was obtained in the usual way and divided into two parts, one of which was placed in a large flask into which nitrogen, after bubbling through water, was passed to a little above the surface of the diluted blood, which was regularly twirled round for 50 to 60 minutes. It is seen in Table 5 that the simple exposure of the diluted blood to nitrogen had negligible effect on the deaminating-enzyme activity of the plasma. In each instance the nitrogen was exposed over the surface for the same period as applied in the perfusion experiment and sample B was plasma obtained from the nitrogen-exposed flask. Sample A was the control plasma obtained from a similar air-exposed flask of diluted perfusion fluid.

In experiment 9 (Table 5) the plasma from the air-exposed perfusion fluid (A) when mixed with adenosine 1:20,000 had no effect on the activity of the system after 30 minutes' incubation. The nitrogen-exposed plasma (B) reduced the activity of the system to 97 p.c. in 30 minutes. In experiment 10 the figures were similar, 40 p.c. reduction of activity for the air-exposed and 40 p.c. reduction of the adenosine system for the nitrogen-exposed, i.e. there was no difference in the deaminating enzyme activity of the two samples. In experiment 11 the air-exposed plasma reduced the deaminating-enzyme activity of the system to 30 p.c. in 30 minutes and of the nitrogen-exposed to 25 p.c. in the same time, again an insignificant difference.

TABLE 5.

Deaminating enzyme activity of plasma air-exposed (A), nitrogen-exposed (B).

Cat No.	p.c. activity after incubation.	
	A	B
9.	100	97
10.	40	40
11.	30	25

The perfusion experiment was begun in the ordinary way with the first portion of the diluted blood obtained and then the air-containing flask was replaced with the nitrogen-containing flask still being delivered with nitrogen and the perfusion-tubing rapidly flushed through up to the organ by opening the tail of the T-piece just proximal to the organ. Perfusion was then continued with the nitrogen-exposed perfusion fluid and perfusion samples collected in the ordinary way.

The details of one experiment (13) were as follows: A liver weighing 101 gm. from a cat weighing 3 kg. was perfused with diluted blood (''air exposed'') for 12 minutes during which 85 ml. of perfusion fluid were collected. Plasma from this sample when mixed with adenosine and incubated for 30 minutes showed an activity after incubation of 43 p.c. Perfusion was continued with nitrogen-exposed diluted blood for six minutes during which time 44 ml. were collected. The diluted plasma so obtained after mixing with adenosine and incubation gave an

Fig. 4.

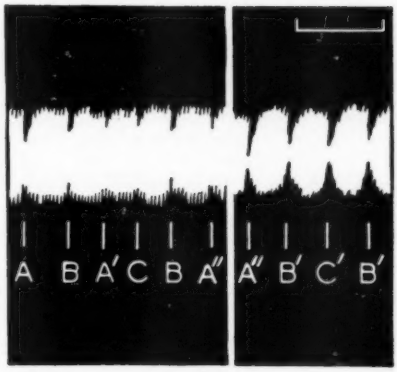

Fig. 5.

Fig. 4. Responses to the auricle of the intact guinea-pig. At A, A′ and A″ responses to 10, 4 and 8γ adenosine. At B response to 0·2 ml. of control plasma mixed with adenosine (1:20,000) and incubated for 30 minutes. At C response to 0·2 ml. of perfused plasma (perfused at 0·7 ml. per minute) mixed with adenosine (1:20,000) and incubated for 30 minutes. At D response to 0·2 ml. of perfused plasma (perfused at 3·9 ml. per minute) mixed with adenosine (1:20,000) and incubated for 30 minutes. Time in minutes. Details in text.

Fig. 5. Responses of the auricles from two guinea-pigs. At A, A′ and A″ responses to 5, 3 and 4γ adenosine. At B response to 0·2 ml. of plasma obtained from cat liver perfused at 7 ml. per minute (''air exposed'') mixed with adenosine (1:20,000) and incubated for 30 minutes. At C response to 0·2 ml. of perfused plasma perfused at 7 ml. per minute obtained from the same liver when ''nitrogen exposed.'' At B′ response to 0·2 ml. of perfused plasma from another cat liver perfused at 7 ml. per minute (''air exposed'') mixed with adenosine (1:20,000) and incubated for 30 minutes. At C′ response to 0·2 ml. perfused plasma perfused at 7 ml. per minute (''air exposed'') mixed with adenosine and incubated for 30 minutes. Time in minutes. Details in text.

activity of 30 p.c. Thus there was a significant reduction in the deaminating enzyme activity of the perfused fluid after exposure to nitrogen compared to exposure with air. The oxygen capacity of the diluted blood was 1·3–2·0 ml./100 ml. The findings in this experiment are shown in Fig. 5. At B the response to air-exposed perfused plasma after incubation is intermediate between 4 and 5γ adenosine. The response at C to the nitrogen-exposed plasma after perfusion after incubation is equal to 3γ adenosine. At A is shown the response to 5γ adenosine. at A′ to 3γ and at A″ to 4γ adenosine.

TABLE 6.

Deaminating enzyme activity of perfused fluid air-exposed (A), and nitrogen-exposed (B).

Cat. No.	Perfusion rate ml. per min.	Wt. liver gm.	p.c. activity after 30 mins'. incubation. A	B
10.	2	150	0	0
11.	4·5	165	20	0
12.	7	105	18	0
13.	7	101	43	30
14.	7	105	33	35*

* No nitrogen used.

In another experiment (No. 11, Table 6) a liver weighing 165 gm. was perfused at a rate of 4·5 ml. per minute and the sample of perfusion fluid collected while the air exposure was continued showed an activity of 20 p.c. after incubation with adenosine. That of the sample when the perfusion fluid was nitrogen exposed was 0, i.e. complete inactivation. In one other experiment (12) the figure for the ''air'' perfusion was 18 p.c. activity and for the nitrogen-exposed 0.

In a control experiment where the liver of a cat was perfused with air-exposed diluted blood at a rate of 7 ml. per minute after the first sample was collected (70 ml. in 10 minutes), the second flask which in this instance was not nitrogen-exposed was raised to replace the first, and the diluted blood was run rapidly through the apparatus to the T-piece as was done with the nitrogen-exposed perfusion and then the perfusion continued with this air-exposed diluted blood. Perfusion was continued for 14 minutes and 100 ml. were collected. The activity of the first sample of perfused plasma after incubation with adenosine was 33 p.c. The activity of the second sample of diluted plasma after incubation (air-exposed) was 35 p.c. These findings are indicated in Fig. 5, where B′ is the response to 1 ml. of the first sample of plasma after incubation and C′ is the response to the second sample of plasma after incubation which is a little greater than that to B′.

DISCUSSION.

In this paper investigations carried out on the condition of experimental muscle ischaemia in the dog have been described. It had already been shown by Nathanson *et al.* (1945) that the fluid obtained following release of the tourniquet producing the ischaemia was toxic in a proportion of cases and was sometimes capable of producing shock in another animal when injected or in the same animal when the fluid was absorbed into the tissues. In this animal, Pope *et al.* (1945), showed that *Cl. welchii* was recovered from most of the exudates they produced following muscle ischaemia and they considered there was a correlation between the toxicity of the fluid and the bacterial content. They suggest that bacterial contamination is the source of the toxic factor. It had been shown (Kellaway and Trethewie, 1941a) that *Cl. welchii A* causes the liberation of deaminating enzyme and adenyl compounds from the isolated perfused liver. It was also shown (Trethewie, 1942b) that in pneumonia there was a correlation between the content of deaminating enzyme in the blood and the malaise of the patient. A similar correlation was found following operation under ether anaesthesia (Trethewie, 1946). In the work recorded in this paper the deaminating enzyme content of the muscle fluid was considerable. Adenosine 1 : 25,000 which in the case of human plasma is normally only about one-half inactivated following 40 minutes' incubation, was always completely inactivated by muscle fluid whether the fluid grew *Cl. welchii* or not. When the concentration of adenosine was raised to 1 : 5,000, following incubation for 30 minutes, a small amount (12·5 p.c.) of adenosine was still active. The concentration of deaminating enzyme in the blood of these animals was very considerably less. In fact, when dog plasma was incubated with adenosine 1 : 25,000 the activity after incubation remained at 50–70 p.c. Thus the deaminating enzyme content cannot be related to simple outpouring of plasma. The fact that there is a considerable increase in deaminating enzyme does not in itself indicate that this is a toxic factor. Further work is necessary in this regard though the correlations mentioned above are suggestive.

It has been shown that a small amount of cardio-depressant substance is present in the centrifuged fluid which is inactivated by a system which inactivates added adenosine. This might well be due to a compound of an adenyl nature. Since muscle is known to contain considerable amounts of adenosine it is possible that it is adenosine liberated from damaged muscle. Green (1943) suggested that the shock developing after release of the tourniquet might be due to the absorption of adenosine triphosphate from damaged muscle. Bollman and Flock (1944) considered this unlikely. Stoner and Green (1945) found a rise in the adenosine equivalent of rabbit blood following lethal limb trauma. In any case occasional cases of shock are associated with a slowing of the pulse rate which is a specific effect produced by adenosine.

The uncentrifuged muscle fluid when boiled has considerable cardio-depressant activity and when tested on the guinea-pig auricle the fluid obtained from experiment 7 in this paper showed not only considerable depression of the

heart beat but, also that the excursion of the heart did not return to the original extent after a degree of immediate recovery.

There was no evidence that either histamine or slow-reacting substance was liberated into the muscle fluid. The absence of liberation of histamine is in confirmation of earlier work.

Perfusion of the liver at a slow rate of flow with diluted blood was associated with an outpouring of deaminating enzyme into the perfused fluid. It was found that the concentration of deaminating enzyme increased as the rate of flow was reduced. It was further shown that this output of deaminating enzyme was increased when the oxygen supply was reduced. It is therefore suggested that the mechanism of liberation of deaminating enzyme with slow rates of flow depends on anoxia. It is to be remembered that in shock many of the phenomena are related to anoxia. In fact it is probable that serious effects of shock are due to the depressed circulation allowing severe results to occur in the organs. Thus this effect becomes a cumulative one and once shock is maintained through any length of time the further damaging effect of the impaired circulation maintains shock. Wiggers and Ingraham (1946) have pointed out that they differentiate three major conditions which may develop from uncomplicated haemorrhage. There is a simple haemorrhagic hypotensive state from which recovery is almost assured without treatment. Next, with more severe haemorrhage, the impending shock state is produced with sustained hypotension and tissue anoxia. If uncorrected this passes into the ingravescent irreversible shock state.

Evidence of the slowed circulation in clinical shock has been shown by circulatory-dye studies. There is no marked change in dye curves. In clinical shock the only significant fact is the prolongation of the mixing time (Noble, Gregerson and co-workers, 1946). Slowing of mixing time is related to the slow circulation of shock. In clinical shock the blood volume following trauma is 30 to 40 p.c. below normal (Noble and Gregerson, 1946). Scott (1946) has indicated that local loss of fluid does not account for death in experimental shock.

It is undoubtedly the case that all the evidence indicates the importance of toxic factors in shock and the following work has indicated some of the complexities of the problem. When shock is produced by haemorrhage after a stage, as has been mentioned, "irreversible" shock occurs, i.e. simple replacement of the blood lost at this stage does not produce recovery of the animal. If, however (Frank, Seligman and Fine, 1946), the blood-flow through the liver is maintained while the remainder of the organism is hypotensive, protection is afforded so that the animal does not pass into the "irreversible" stage. Eleven of twelve dogs so treated recovered. Fifteen of seventeen dogs not so protected died. Therefore the loss of liver integrity is a significant factor in collapse in advanced haemorrhagic shock and the maintenance of liver function is paramount. Other organs were not considered related.

It has further been shown (Shorr, Zweifach and Furchgott, 1945) that vaso-depressor material (V.D.M.) liberated in shock and assessed by the reactivity of the meso-appendix is produced by the liver only after the "irreversible" stage. Thus the "irreversible" state is related to production of vaso-depressor material by the liver in shock. V.D.M. is found in the limbs during the hyper-reactive stage and it is absent from the circulation possibly because it is masked by prodigious amounts of vaso-excitor material (V.E.M.), This V.D.M. is probably produced by muscle. In the hyper-reactive phase of shock the kidney is important. The terminal vascular bed is acted on by V.E.M. during this phase and the kidney is probably the source of the humoral V.E.M. After renal occlusion profound shock is produced much more rapidly than in normal animals and thus in haemorrhagic shock less blood loss is tolerated.

TABLE 7.

Active substance liberated.

Condition or agent.	Histamine.	S.R.S.	Adenyl Cpd.	Deaminating enzyme.	Acetyl-choline.	Adrenalin.	Potassium.	Leuko-taxine.	Leucocy-tosis factor.	Necrosin.		"Aub's" Sub-stance.	Anti-coagu-lant.
										Necrosin.	Pyrexin.		
Anaphyl-axis	+ Bartosch, Feldberg & Nagel (1932) Dragstedt & Gebauer-fuelnegg (1932)	+ Kellaway & Trethewie (1940b)	— Kellaway & Trethewie (1940c)										+ Jaques & Waters (1941)
Venom Cobra	+ Feldberg & Kellaway (1938b)	+ Feldberg & Kellaway (1938b)	+ Kellaway & Trethewie (1940a)	+ Kellaway & Trethewie (1940a)	+ Gautrelet & Corteggiani (1938)								
Copper head	+ Feldberg & Kellaway (1937a)	+ Feldberg & Kellaway (1937a)											
Black Snake & Death Adder	+ Trethewie (1939)	+ Trethewie (1939)											
Bee	+ Feldberg & Kellaway (1937b)					+ Feldberg (1937a)							
Trypsin	+ Rocha e Silva (1940)	+ Trethewie (1942a)	+ Trethewie (1942a)	+ Trethewie (1942a)				+ Cullumbine & Rydon (1946)					
Peptone	+ Feldberg & O'Connor (1937)												+ Wilander (1939)

TABLE 7 (continued).

TABLE 7 (continued).

Condition or agent.	Histamine.	S.R.S.	Adenyl Cpd.	Deaminating enzyme.	Acetyl-choline.	Adrenalin.	Potassium.	Leuko-taxine.	Leucocy-tosis factor.	Necrosin.		"Aub's" Substance.	Anti-coagulant.
										Necrosin.	Pyrexin.		
Heat	+ Kellaway & Rawlinson (1944)		+ Bennet & Drury (1937)					+ Menkin (1936) + Cullumbine & Rydon (1946)					
Mercuric Chloride	+ Feldberg & Kellaway (1938a)	— Feldberg & Kellaway (1938a)											
Radiant energy	+ Kellaway Holden, & Trethewie (1938)	— Kellaway, Holden & Trethewie (1938)											
Photo dynamic action	+ Kellaway & Trethewie (1939a)	— Kellaway & Trethewie (1939a)											
Toxin Staphylococcal	+ Feldberg & Keogh (1937)												
	+ Feldberg & Kellaway (1938a)	+ Feldberg & Kellaway (1938a)											
Cl. *welchii* A	— Kellaway & Trethewie (1941a)	— Kellaway & Trethewie (1941a)	+ Kellaway & Trethewie (1941a)	+ Kellaway & Trethewie (1941a)									
B	+ Kellaway & Trethewie (1941b)	— Kellaway & Trethewie (1941b)	+ Kellaway & Trethewie (1941b)	+ Kellaway & Trethewie (1941b)									
C	— Kellaway & Trethewie (1941b)	+ Kellaway & Trethewie (1941b)	+ Kellaway & Trethewie (1941b)	+ Kellaway & Trethewie (1941b)									
D	+ Kellaway Trethewie & Turner (1940)	— Kellaway, Trethewie & Turner (1940)	+ Kellaway, Trethewie & Turner (1940)	+ Kellaway, Trethewie & Turner (1940)									
Septique	+ Kellaway, Reid & Trethewie (1941)	— Kellaway, Reid & Trethewie (1941)	+ Kellaway, Reid & Trethewie (1941)	+ Kellaway, Reid & Trethewie (1941)									
Pneumococcal Extract "Inflammation"			+ Trethewie (1942b)	+ Trethewie (1942b)				+ Menkin (1936) (1943a)	+ Menkin (1940) (1943a)	+ Menkin (1943a) (1943b) (1943c) (1944)			

TABLE 7 (continued).

Condition or agent.	Histamine.	S.R.S.	Adenyl Cpd.	Deaminating enzyme.	Acetyl-choline.	Adrenalin.	Potassium.	Leuko-taxine.	Leucocy-tosis factor.	Necrosin. Necrosin.	Necrosin. Pyrexin.	"Aub's" Sub-stance.	Anti-coagu-lant.
Anaesthetics N_2O,C_2H_4 90, 95 p.c.	+ Kellaway & Trethewie (1939b)	— Kellaway & Trethewie (1939b)											
Ethyl-chloride 30, 25 p.c.	+ Kellaway & Trethewie (1939b)	+ Kellaway & Trethewie (1939)											
Ether 16, 8 p.c.	+ Kellaway & Trethewie (1939b)	+ Kellaway & Trethewie (1939b)		+ Trethewie (1946)									
Chloroform 4 p.c.	+ Garan (1938) Kellaway & Trethewie (1939b)	+ Kellaway & Trethewie (1939b)	+ Trethewie (1941)	+ Trethewie (1941)	+ Trethewie (1941)								
Spinal				— Trethewie (1946)									
Phosgene	+ Trethewie (1947a)	+ Trethewie (1947a)						+ Trethewie (1947b)					
Hydro-carbons	+ Garan (1938)												
Turpentine								+ Menkin (1936)	+ Menkin (1943a)	+ Menkin (1943a)			
Pneumonia	+ Trethewie (1945a) (1945b)	+ Trethewie (1945a) (1945b)		+ Trethewie (1942b)			+ Trethewie & Cowan (1947)						
Shock Ischaemic	— Trethewie (1947c)	— Trethewie (1947c)		+ Trethewie (1947c)								+ Aub *et al.* (1945)	
Anoxia				+ Trethewie (1947c)									
Haemolysis				+ Trethewie (1946)						+ Menkin (1943b)			

The importance of pursuing studies of this nature is realized if one regards the problem of tissue injury as one in which many factors operate. There are local nervous effects. Other harmful effects are produced by the liberation of pharmacologically active substances most of which are already present preformed in the tissues. Some are determined, e.g. the coronary constrictor effect of the *Cl. welchii* group of toxins (Buttle and Trevan, 1928), by direct effects of the injurious agent. Finally, other effects are produced in a secondary way as for example the anoxia of shock (Trethewie, 1947c). If now we endeavour to discover the substances concerned in the development of the pattern of the signs and symptoms of the sick organism we find that we may draw up a Table and so relate different conditions to specific principles. This has been done in Table 7. It is noted that in anaphylaxis histamine is liberated (Bartosch, Feldberg and Nagel, 1932; Dragstedt and Gebauerfuelnegg, 1932), a slow-reacting substance is liberated (Kellaway and Trethewie, 1940b), adenyl compound is not liberated (Kellaway and Trethewie, 1940c) and anticoagulant (heparin) is liberated (Jaques and Waters, 1941). With the venoms (Table 7) there is a wider variety of substances liberated, e.g. histamine (Feldberg and Kellaway, 1937a, 1937b, 1938b; Trethewie, 1939), slow-reacting substance (Feldberg and Kellaway, 1937a, 1938b; Trethewie, 1939), lysocythin (Feldberg and Kellaway, 1938b), adenyl compounds (Kellaway and Trethewie, 1940a), deaminating enzyme (Kellaway and Trethewie, 1940a) and acetylcholine (Gautrelet and Corteggiani, 1938). Trypsin similarly liberates a wider variety of substances (Rocha e Silva, 1940; Trethewie, 1942a; Cullumbine and Rydon, 1946) probably because it also is of an enzyme nature. Peptone as in anaphylaxis causes the liberation of histamine (Feldberg and O'Connor, 1937) and anticoagulant (heparin) (Wilander, 1939) showing an activity similiar to anaphylaxis, a phenomenon to be expected in view of the clinical responses. Heat (Kellaway and Rawlinson, 1944), mercuric chloride (Feldberg and Kellaway, 1938a), radiant energy (Kellaway, Holden and Trethewie, 1938) and photodynamic action (Kellaway and Trethewie, 1939a) liberate histamine and the mechanism of this action may be similar and of a coagulative type. The bacterial toxins, e.g. the *Cl. welchii* group, indicate a difference in the activity of the toxins with regard to the liberation of histamine (Table 7) and S.R.S. This is probably related to the different constitution of the toxin of different types (Kellaway and Trethewie, 1941a, 1941b; Kellaway, Trethewie and Turner, 1940). In inflammation Menkin (1936, 1940, 1943a, 1943b, 1943c, 1944) has shown the liberation of leukotaxine, necrosin (necrosin and pyrexin) and a leukocytosis factor. He believes that this mechanism is similar when produced by a wide variety of injurious agents. Among the anaesthetics given we have a difference with regard to the liberation of histamine and S.R.S. and also with regard to the sequential liberation of them when both are liberated. Phosgene also causes the liberation of histamine and S.R.S. (Trethewie, 1947a). In pneumonia histamine, S.R.S., deaminating enzyme and potassium are liberated (Trethewie, 1942b, 1945a, 1945b; Trethewie and Cowan, 1947). In shock (ischaemia) neither histamine nor S.R.S. is liberated (Trethewie, 1947c). Deaminating enzyme is liberated (Trethewie, 1947c) as is also a toxic factor in the protein fraction (Aub *et al.*, 1945). In other forms of shock similarly a vascular depressant occurs (Shorr, Zweifach and Furchgott, 1945). In anoxia deaminating enzyme is liberated (Trethewie, 1947c). In haemolysis deaminating enzyme (Trethewie, 1946) and necrosin (Menkin, 1943b) are liberated.

There are a number of substances which are inhibitory to the pharmacological substances liberated in these forms of tissue injury. Antagonists to histamine are phenol-ethers of amino alcohol, aniline derivatives and amino-pyridine (Bovet and Walthert, 1944) (Table 8). There are other substances which break down

histamine rather than being simply inhibitory. Similarly there are inhibitors against S.R.S. (S.R.S. itself and CO_2). Some inhibitors are toxic and therefore are not suitable. Potassium is inhibited in its toxic effect by glucose, and leukotaxine by adrenal cortex. The importance of therapy suggested along these lines was shown by Rosenthal and Brown (1940) who were able to save animals from death following the administration of the anaphylactic antigen with pre-treatment of the animal with phenol-ether. In phosgene poisoning it was shown (Trethewie, 1947a) that a compound of this type greatly reduced the mortality in guinea-pigs when the substance was given some minutes after exposure.

TABLE 8.

"Inhibitors" and "inactivators" of pharmacologically active substances.

Active substance.	"Inhibitor."
Histamine	Thymoxyethyl diethylamine (phenol ether). Aniline derivative. Amino-pyridine. CO_2 $FeSO_4$ and vitamin C.
S.R.S.	S.R.S. Lysocythin (toxic.) CO_2
Adenyl Compound	Deaminating enzyme.
Deaminating enzyme	Cyanide.
Adrenaline	Ergotamine.
Acetylcholine	Atropine.
Potassium	Glucose (partial). Sodium.
Aub's substance	?
Leukotaxine	Adrenal cortex.
Leucocytosis factor	?
Pyrexin	?
Anticoagulant	Prothrombin.

SUMMARY.

It has been shown that deaminating enzyme is liberated from ischaemic muscle after the circulation has been renewed.

Cardio-depressant substance was detected in the centrifuged fluid obtained from the ischaemic muscle and larger amounts in the sediment.

Neither histamine nor S.R.S. was found to be liberated from muscle.

Deaminating enzyme was liberated from the isolated liver of the dog and cat when perfused with diluted heparinized blood at a slow rate of flow. As the rate of flow was reduced the output of enzyme increased.

When the perfusing diluted blood was nitrogen-exposed prior to perfusion there was an increased output of deaminating enzyme from the perfused liver of the cat.

The significance of these findings is discussed.

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A COMPARISON OF ABILITY TO WORK WITH LOW (2·7–2·8 IN.) AND HIGH (3·2–3·3 IN.) RESISTANCE RESPIRATOR CONTAINERS[1]

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It was desired to determine if there is any reduction in the ability to work with higher resistance containers (3·2–3·3 in. water) compared with the lower resistance container (2·7–2·8 in. water).

Difficulties arise in field tests in that if a series of obstacles are introduced it is not easy to duplicate exactly the same pathway over the obstacles and a varying amount of effort may be expended in successive runs so that slight but significant differences may be missed. For this reason the experiments were divided into two types. In the first, work done on a bicycle ergometer was read directly. In the second, races were run over a 305 yards "U"-shaped course marked out by a single white line. The bend in the course passed over a steep rise. It is the effort of which each individual is capable, rather the blood pressure or pulse change, that is important in assessment.

METHOD.

Resistance Measurements.

Resistances (all at 3 cub. ft. per minute rate of flow) were measured in inches of water directly from a U-tube water manometer placed in circuit with a flow-meter (Fig. 1) calibrated from the meter measuring volume flow and from a stop-watch. In a group of six containers, the resistance of each of which was measured twice at an interval of 15 minutes and in which the rate of flow was lowered between each estimation by adjusting the needle valve (v) (Fig. 1) the resistance varied from 0·00 to 0·04 in. water with a standard deviation of 0·03 in. In a series of six containers which were placed in a cupboard for five months and not used, the variation after that time was greater being from 0·01 to 0·08 in.

Before the experiment six "low" resistance containers were selected and paired with six "high" resistance containers as shown in Table 1. The subjects using each pair are shown in the first column. These numbers correspond to the facepiece numbers.

TABLE 1.

Resistance (in. water) of containers—rate of flow 3 cubic ft. per minute.

Subject No.	Low.		High.	Difference.
2, 11	2·78	paired with	3·26	0·48
3, 12	2·70		3·19	0·49
1, 6	2·72		3·22	0·50
4, 10	2·78		3·33	0·55
7, 9	2·65		3·24	0·59
5, 8	2·72		3·32	0·60

After the whole experiment was concluded the differences in resistances were as follows: 0·60, 0·57, 0·59, 0·59, 0·33, 0·65 respectively, for the order in Table 1. All of the containers except one in each group had increased slightly in resistance and the variation was greater than

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either of the variations described for immediate (15 minutes) retest and delayed (5 months) retest without use. Evidently use was responsible for the rise. The average difference before the experiment was 0·54 in. and after the experiment 0·56 in. water at 3 cub. ft. per minute. The resistances of the facepieces (measured when hanging vertically with air flowing away from facepiece down tube) and outlet valves are shown in Table 2.

TABLE 2.

Facepiece No.	Resistance of facepiece and tube (in. water).	Resistance of outlet valve (in. water).
1.	0·64	0·82
2.	0·63	0·84
3.	0·65	0·79
4.	0·60	0·85
5.	0·58	0·83
6.	0·64	0·77
7.	0·63	0·81
8.	0·58	0·79
9.	0·63	0·79
10.	0·61	0·86
11.	0·62	0·81
12.	0·64	0·83

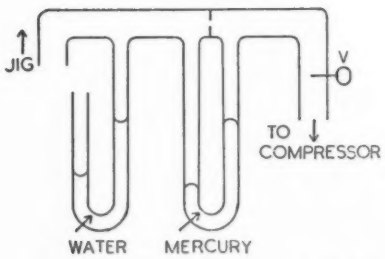

Fig. 1. Diagram of apparatus for measuring resistance.

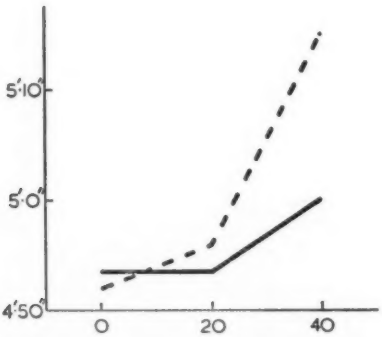

Fig. 2. Ordinates—time for race in minutes. Abscissae—interval between races in minutes. The continuous line indicates the times for the stronger team and the interrupted line the times for the weaker team.

Bicycle Ergometer.

A bicycle ergometer (Martin, 1914) was assembled with a spring attachment (Porton, 1923) connected to one end of the canvas belting. The spring reading was measured by a pointer writing on a slowly revolving smoked drum which was attached to the spring by a cord passing over pulleys. By means of another cord and pulleys, 0·5, 1·0 and 1·5 lb. weights were attached to the spring after detaching the belting and so the tension in the spring was easily measured (see Fig 3). Distances were measured on the varnished paper with dividers and a metal rule. The average figure of four readings was 4·4 mm. = 0·5 lb., 8·8 mm. = 1 lb., 13·3 mm. = 1·5 lb. The weight of the buckle and belt, plus the weight suspended on the belt was 7·17 lb. The frictional resistance was not perfectly constant from day to day, there being a gradual increase in resistance as the experiment proceeded, possibly due to a slight increase in the folding of the edges of the belting (which had a $\frac{1}{4}$ in. margin) round the rim of the wheel. Weather changes may also have varied the friction. In general the tension in the spring was a little greater with the first run on each day and diminished slightly with use during the day. This means the friction slightly increased. The figures for the friction in each experiment will be found in Table 3. The circumference of the wheel was 5 ft. $4\frac{3}{8}$ in.

TABLE 3.

Work done on Bicycle Ergometer.

Subject.	Av. diff. resistance in. H_2O.	Container resistance.	Frictional resistance lb.	No. of revolutions.	Work done rev. lb.
1.		High	6·00	4,335	26,010
		Low	5·90	4,610	27,199
	·55	High	5·92	4,495	26,610·4
2.		High	5·85	*4,174	24,417·9
		Low	5·88	4,332	25,472·16
	·54	High	5·84	4,289	25,047·76
3.		High	5·87	4,106	24,102·22
		Low	5·95	4,505	26,808·75
	·53	High	5·97	4,421	26,393·37
4.		High	5·93	3,708	21,988·44
		Low	5·92	3,940	23,324·8
	·57	High	5·97	†3,102	18,518·94
5.		Low	5·99	4,398	26,344·02
		High	6·03	4,134	24,928·02
	·63	Low	5·99	4,476	26,813·22
6.		Low	5·90	4,325	25,517·5
		High	5·92	4,245	25,130·4
	·55	Low	5·93	4,256	25,238·08
7.		High	6·13	4,099	25,116·87
		Low	6·07	4,160	25,251·2
	·46	High	6·07	4,158	25,239·06
8.		High	6·05	4,253	25,730·45
		Low	6·06	4,534	27,476·04
	·63	High	6·12	4,364	26,707·68
9.		High	5·95	4,713	28,042·35
		Low	5·97	4,874	29,097·78
	·46	High	6·07	4,734	28,735·38
10.		Low	5·98	4,601	27,513·98
		High	5·95	4,362	25,983·9
	·57	Low	6·01	4,694	28,210·94
11.		Low	5·87	4,144	24,325·28
		High	5·87	4,160	24,419·2
	·54	Low	5·94	4,301	25,547·94
12.		High	5·87	4,203	24,671·61
		Low	5·87	4,300	25,241
	·53	High	5·89	4,283	25,226·87

The subjects undertaking the short bursts of maximal work (4 minutes) were students. but for all other investigations—20 minutes' maximal effort, races, and respiratory pressure changes—the subjects were soldiers from an employment company who were used to heavy work. The soldiers wore light Australian battle-dress (long trousers and shirts) respirator at the alert with facepiece adjusted, equipment and a pack weighing 9–11 lb. Each individual used his own pack, the contents of which were not altered during the entire experiment, and it was weighed before each test. The variation in weight was 1 to 2 oz., occasionally slightly more. The blood pressure (systolic and diastolic) was measured twice with the stethoscope and a mercury manometer at an interval of five minutes before each test, and again immediately on cessation of the test, and again 28 minutes later after sitting quietly. The pulse was similarly measured, the pulse reading immediately preceding the blood pressure reading. A rigid training programme lasting one to two weeks (varying with each individual) and maintained throughout the duration of the experiment, was undertaken during which periods of work done on the ergometer were increased from 10 to 15 and finally to 20 minutes, so that the subjects could hardly work any longer at the expiration of the 20 minutes. Finally the experiment was undertaken with the individual wearing on the first day one of the paired (low or high) containers, on the second day the other container, and on the third day the first container was used again. Work was done at the same time each day for each individual. This, in itself, ensured better agreement in the friction in the belting on each occasion, and also, as in weighing by vibrations,

allows a better comparison of the two types of container, should the state of training of the individual be improving or diminishing. Early nights and no alcohol were insisted upon during the tests.

RESULTS.

Bursts of Maximal Effort for 4 Minutes.

Preliminary training was undertaken by the subjects and in the first experiments maximal effort was exerted which usually could be sustained for not longer than four minutes. At first the facepiece was worn. then the facepiece and container, and trials were made on successive days at the same time for each of the twelve subjects. For the experiment six subjects rode on three alternate days wearing a high resistance, then the paired low resistance container, and finally the high resistance container again. Maximal effort was exerted for four minutes timed by the stopwatch. Each subject had his own facepiece. The other six subjects alternated with a low resistance container, then a high and again the low resistance container. The average of the first and third runs was compared with the second run and the average increase in work done per subject as 100 revolution pounds (equal to 100 turns of the wheel against a friction of 1 lb.) for the low container as compared with the high was 0·71. The individual increases were 1·3, 7·5, —1·0, 2·3, 2·0, 0·5, —2·0, 3·6 —3·6, —2·4, —0·8, 1·1. This gives a standard deviation of 3 and for a mean difference of 0·71 (obtained) 80 pairs (Snedecor "Z" test, p. 37) would be required for comparison. Since only twelve pairs were tested the difference is not significant.

Sustained Effort to "Near Exhaustion" in 20 Minutes.

It was recognized that while there may be no significant difference for a burst of work lasting four minutes it might be that more prolonged exertion might show a difference in work done with the varying resistance.

(a) The findings comparing the work done on successive days with each type of container are shown in Table 3.

In the case of Subject 2 (see Table 3*) he was so exhausted after 19 minutes using the high resistance container on this occasion that he was unable to continue. The other two readings for him were for 20 minutes' work. At a later stage (see respiratory pressure measurement) this same subject was able to work for 19 minutes only with the high resistance container (two occasions) and for 19′ 30″ only with the low resistance container (on one occasion). In the case of Subject 4 (see Table 3†) he was so exhausted after 15′ 34″ with the high resistance container that he was unable to continue.

TABLE 4.

Subject.	Increase in work rev. lb. per man with low resistance container.
1.	888·8
2.	739·33
3.	1,556·95
4.	3,071·11
5.	1,650·6
6.	247·39
7.	73·23
8.	1,256·87
9.	708·91
10.	1,878·56
11.	517·41
12.	291·76

In Table 4 increase in work done as rev. lb. per subject for the high container are recorded, e.g. Subject 1 averaged 26,310·2 rev. lb. with the high resistance container and performed 27,199 rev. lb. with the low resistance container, giving a difference of 888·8 rev. lb. work done. In every instance more work was done with the low resistance container.

The mean difference in the work done was 1,073·41 rev. lb., giving an increase of work with the low resistance container of 3·5 p.c. or a decrease of work with the high resistance container of 3·4 p.c. The standard deviation of the increases in work done is 858·5 and for a mean difference of 1,073·41 this finding is significant (20:1) if only 5·1 pairs are compared ("Z" test) and highly significant (100: 1) if only 8·3 pairs are compared. Since we have compared more, namely twelve pairs, the findings are highly significant.

Changes in the pulse. The changes in the pulse with each type of container are shown in Table 5. The average pulse before the exertion was taken as the initial pulse and where two runs were made with a container the figures before and after the exertion were averaged for comparison.

TABLE 5.

Pulse increase (per minute) following exertion.

Subject.	Immediately after exertion high container.	Immediately after exertion low container.	28 mins. after exertion. high container.	28 mins. after exertion. low container.
1.	79	88	32	24
2.	75	90	19	26
3.	64	62	28	26
4.	68	65	23	13
5.	44	75	11	43
6.	42	50	9	14
7.	67	83	26	32
8.	71	74	18	30
9.	84	86	20	14
10.	94	98	38	27
11.	77	79	25	19
12.	76	72	28	21
Average	70	77	23	24

The pulse rose 70 beats per minute on the average with the high resistance container and 77 beats per minute with the low container, and 28 minutes after exertion the rises were 23 and 24 respectively. A considerable difference is noted with Subject 5 in the rises in pulse immediately after exertion, 44 and 75. This is partly accounted for by the fact that with the first, his initial pulse was high, 106; while with the second it was 80. Apart from emotional causes I could not account for the first high initial pulse.

Changes in Blood Pressure. The changes in systolic and diastolic blood pressure 1 minute and 29 minutes after the exertion are shown in Table 6. The blood pressure changes were more variable than the pulse changes, e.g. in column 1 with Subject 2, 20 is averaged from 9 and 31; with Subject 7, 1 is averaged from +18 and —16, and with Subject 9, 12 is averaged from 23 and 1.

TABLE 6.

Blood pressure changes mm. Hg.

Subject.	Systolic. (A fall is denoted by —). Minutes after exertion. 1 high.	1 low.	29 high.	29 low.	Diastolic. Minutes after exertion. 1 high.	1 low.	29 high.	29 low.
1.	6·5	24	—12·5	—11	— 7	— 4	0	+3
2.	20	14	—21	—20	— 2	— 6	— 3·5	—6
3.	10·5	14·5	+ 2	+ 1·5	— 3	— 5	+ 5	+ 14
4.	—1·5	5·5	—16·5	+ 4·5	+ 2	—10	—14·5	—13
5.	28	2	—22	—27·5	— 8	+ 6	+ 4	—13
6.	15	10·5	— 6	—16·5	—11	—10	—18	—16
7.	1	13	—10	—18	+ 1	+ 9	— 7	+11
8.	15	20·5	—14	— 6·5	— 4	+ 0·5	+ 3	+2·5
9.	12	9·5	—21	—21·5	+ 2	+ 4	—11	— 4
10.	— 0·5	9	—20·5	—19·5	+ 4	—11	— 6	+7
11.	— 4	— 1·5	—12	—13	0	— 4	+ 2	—3·5
12.	26	25	—12	—15	+11	+10·5	+ 2	+4·5
Av.	+11	+12	—14	—14	— 1·3	— 1·7	— 3·7	—1·3

There is a similar average increase of blood pressure with each type of container (+11 and +12 mm. Hg.) while the falls after the exertion, which were notable, were 14 mm. Hg. with each. The diastolic fall was slight, and was three times as great with the high resistance container (3·7 mm. Hg.) but again there was considerable variation in the changes.

Race Times.

Running in pairs. Races were run in pairs each subject wearing light Australian battle-dress (long trousers, shirt, no jacket), haversack, equipment and bayonet fixed. One subject ran just to one side of the line and the second just to the other side of the line. As before, training was undertaken, this time for two to three weeks. An attempt was made to pair subjects who had approximately equal running time.

These races were run at intervals of 20 minutes and containers were alternated as before. Six subjects ran with low, high and low containers, and the other six with high, low and high containers. The times were as follows in Table 7.

TABLE 7.

Races in pairs. (Time in seconds.)

Subject.	Container.				Averages.		Lessening of time with low container.
	high.	low.	high.	low.	high.	low.	
1.	57·6	60	67·1		62·4	60	2·4
2.	56·6	54·7	57·6		57·1	54·7	2·4
3.	64·3	65·4	66·7		65·5	65·4	0·1
4.		66·1	66·3	73	69·6	66·3	3·3
5.		67·5	67·9	67·9	67·9	67·7	0·2
6.		53·2	57	59·6	57	56·4	0·6
7.	60·2	57·8	60·6		60·4	57·8	2·6
8.		60·6	65·1	62·8	65·1	61·7	3·4
9.	60·3	60·4	65·8		63·1	60·4	2·7
10.		65·4	66·2	59·8	66·2	62·6	3·6
11.		58·2	58·3	59·2	58·3	58·7	—0·4
12.	57·4	58·2	60·6		59	58·2	0·8

The mean difference for the times is 1·8 seconds and the standard deviation is 1·4. Applying the "Z" test five pairs would require comparison for such figures to be significant (20 to 1) and 8·2 pairs for it to be highly significant (100 to 1). Since more were compared, namely, 12 pairs, the result is highly significant and the lower container allows a more rapid course to be run.

With the first four subjects used (1, 4, 6 and 10), even after training was considered adequate, there was a considerable variation in the time of the first and third run with the same container, the change being 9·5, 6·9, 6·4 and 5·6 seconds. Though the alternation of containers for these runs would diminish to a considerable extent the change due to tiring, it was considered advisable for the remaining eight to prolong the training for a further period till the first and third runs might be closer in time. This was achieved, the variations in order being for Nos. 2, 3, 7, 9, 12, 4, 5 and 8: 1·0, 2·4, 0·4, 5·5, 3·2, 1·0, 0·4 and 2·2 seconds.

Running in relay teams. One subject of each pair (Table 1) was picked by drawing headed and non-headed matches. Two of the subjects had a "cold" and it seemed wise to exclude them and use teams of five each while the individuals were trained. One team wore low resistance containers while the other team wore high resistance containers and they exchanged containers for the second run 20 minutes later, and in the third run, 20 minutes later again, used the original containers. The time for the whole course was measured by the stop-watch; the results in one relay race series are shown in Table 8.

TABLE 8.

	Team 1.	Team 2.
Race 1	High containers. time 4′ 53·8″	Low containers. time 4′ 52·6″
Race 2	Low containers. time 4′ 53·4″	High containers. time 4′ 55·9″
Race 3	High containers. time 5′ 0·1″	Low containers. time 5′ 15·1″

Team 2 (who on their previous averages were 1·4 seconds per man slower than Team 1 with low resistance containers) were able to win the first race by a narrow margin (1·2 seconds) when Team 1 wore high resistance containers and Team 2 low resistance containers. Team 2 were very exhilarated by their win, since they believed Team 1 included the faster runners. In the second race Team 2 was closely defeated by Team 1 (2·5 seconds) when Team 2 wore the high and Team 1 the low resistance containers. Team 2, however, who had been running better than usual obviously had been more exhausted than Team 1, since they were 22·5 seconds slower in their third than their first run, while the other team had a difference of 6·3 seconds only. Their appearance at the end of the race also bore this out. This is an inherent difficulty in relay races, where one team is definitely stronger than the other. In the other series of relay races two of the subjects were required for an N.C.O's course, and their places were taken by the two subjects who had had a "cold" and were now better (one week later). In this instance Team 2 averaged, on previous performance, 2·7 seconds less per man than Team 1. They ran

first with high resistance containers. The discrepancy between their first and third runs was greater (30·7 seconds) as might be expected, since they had to exert themselves more than ever in the first run to keep up with the better No. 1 Team. It is also possible that when a runner in the better team is well ahead, he may not fully extend himself. Also errors between the finish of the course by one man, and the starting of another are difficult to exclude in a relay race. Owing to the considerable exhaustion of the weaker team due largely to an increased effort to keep up with the stronger team, it is not reasonable to compare their times by alternation since the third race was so slow. This is shown graphically in Fig. 2.

The times of the stronger Team are shown in Table 9.

TABLE 9.

	Runs with: high container.	low.	high.	low.
1st day	4′ 53·8″	4′ 53·4″	5′ 0·1″	
2nd day		4′ 45·4″	5′ 3·5″	5′ 5·4″

The average time on the first day was 3·6 seconds faster with the low resistance container and on the second day 8·1 seconds faster with the same type container.

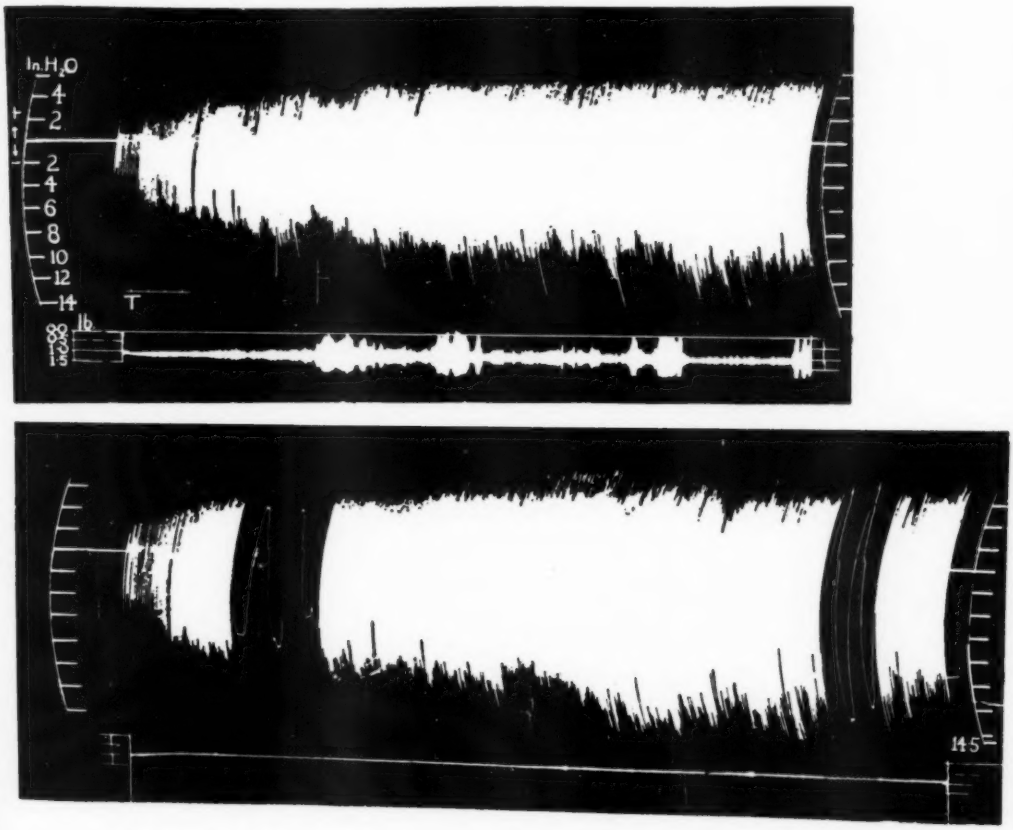

Fig. 3. Subject 1. Respiratory pressure changes. (Reduced ⅓ approx.). Pressures in ins. water. Below zero—inspiration, above zero—expiration. Upper tracing—low resistance container used. Lower tracing—high resistance container used. Time (T) in half minutes. Drum speed changed to fast twice in lower tracing for sampling respiratory graph shape. Spring tension measured in lb.

Measurement of Respiratory Pressures.

In an attempt to obtain some idea of the force expended in respiration, the suction pressures developed performing work with these containers was determined. Three subjects were kept in training for the bicycle ergometer and a tube was connected from the microphone site to a Marey Tambour over which dental dam was tightly stretched. Pressures were calibrated by means of a U-tube and it was found that the scale was almost linear. The suction pressure developed increased as the exercise continued. It is seen (Fig. 3) that with the high resistance container Subject 1 developed a pressure of —12 to —14 in. water (and even greater) during the last eight minutes of the work. The same subject developed a suction of —12 in. water only with the low resistance container. In the case of the second Subject, a suction of —16 in. water was developed at the end of the work with the high resistance container and only —13 in. water with the low resistance container (Fig. 4). The third Subject developed a suction of over —18 in. water which was the limit of the reading of the tambour. In the case of the high resistance container this was reached much sooner than with the low resistance container. A pressure of —16 in. water was developed with the high container in 9–10 minutes in one instance, and 10 minutes in the other, but it was reached only after 15 minutes with the low resistance container. It must be remembered that inertia and pressure-wave effects are introduced in this method of recording.

The outline of the area was marked out with tracing paper and the area measured with a planimeter. This area is approximately proportional to the force expended in respiration. The findings are recorded in Table 10. In this Table the work done (rev. lb.) is also included.

The areas for Subject 1 can be compared directly, but for Subjects 6 and 2, a correction was required for the tambour recording, since the scale was not identical on different days. The tambour had been altered in an endeavour to record the higher suction pressure developed by Subject 2. With Subject 6, 10 in. water pressure = 6·05 cm. with the high container and the same pressure = 5·5 cm. with the low resistance container, (third experiment). An approximation was then made by multiplying the area for the low container by 1·1. This was done and the Table contains the corrected figures. If the scale were not almost linear this would be untenable.

TABLE 10.

Subject.	Container.	Area expiration sq. in.	Area inspiration sq. in.	Work done rev. lb.
1.	Low	9·0	16·4	26,499·6
	High	10·7	20·6	26,528·37
	Low	10·8	14·7	27,750
6.	Low	8·4	20·9	27,125
	High	8·7	26·9	26,474
	Low	8·7	21·6	25,965·3
2.	High*	9·6	36·1	27,180·42
	Low†	9·7	30·1	28,247·2
	High*	10·7	36·2	27,556·65

* Work only able to be maintained for 19 minutes.
† For only 19 minutes 30 seconds.

By comparing the areas in Table 10 it is seen that there is little difference in the pressure developed on expiration, the pressure being 4 p.c. more with the high resistance container. The inspiratory pressure averaged 24 p.c. greater with the high resistance container. The pressure difference in the container at 3 cub. ft. per minute rate of flow is 20 p.c. When the facepiece is included the pressure difference becomes 17 p.c. This difference (24 p.c.–17 p.c.) if it be significant may be attributable to turbulence of flow since it is evident that for part of the respiratory cycle the inspiratory rate of flow is greater than 3 cub. ft. per minute.

Pulse and Blood Pressure Changes.

From Table 11 it is seen that there was little difference in the pulse changes and in the systolic pressure changes. The diastolic pressure fall was a little greater with the high resistance container 29 minutes after the exercise: —13 compared to —5 mm. Hg. This average does not include Subject 2. The systolic pressure fall 29 minutes after the exercise was 8 mm. Hg. with the low, and 4 mm. Hg. with the high resistance container.

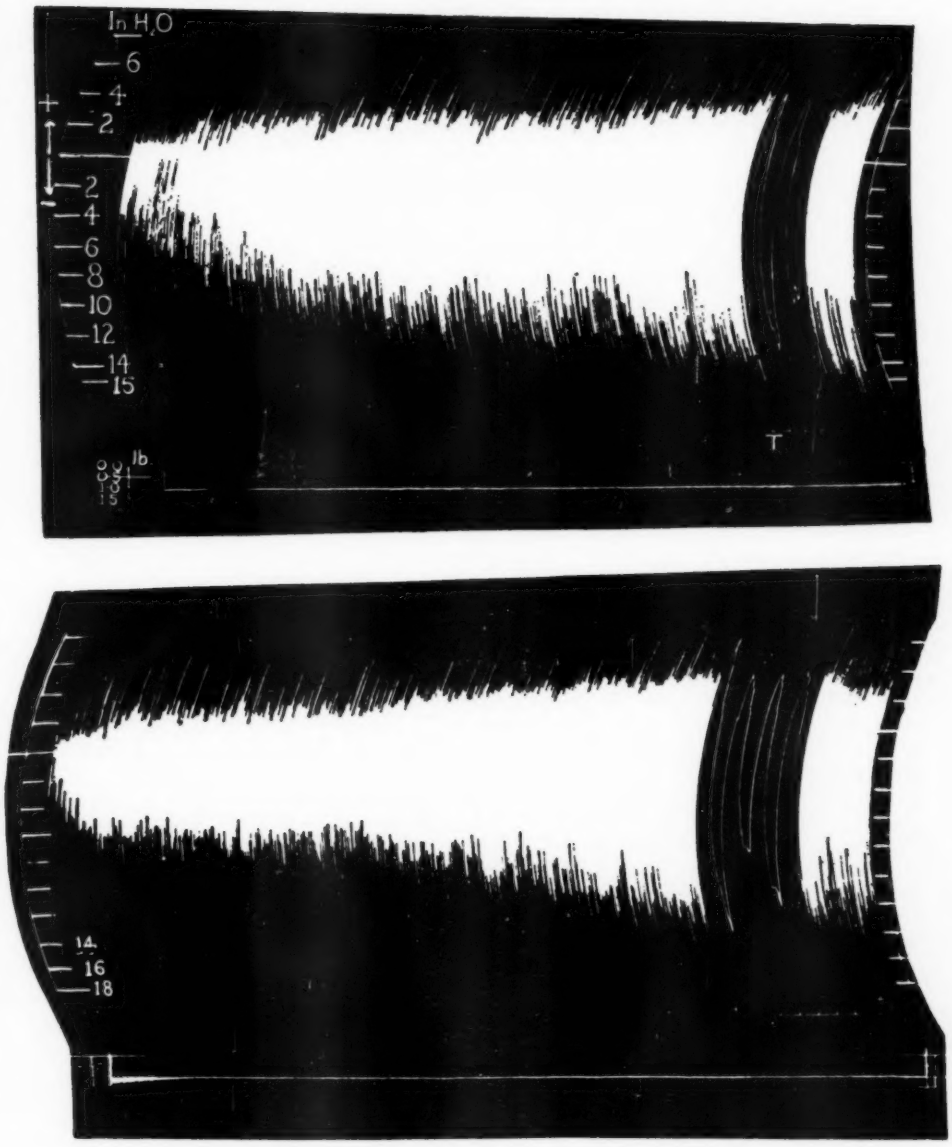

Fig. 4. Subject 6. Upper tracing—respiratory pressure with high resistance container. Lower tracing—respiratory pressure with low resistance container. Drum speed changed at 18 minutes for sampling respiratory effort. Ordinates and abscissae as in Fig. 3.

TABLE 11.

Pulse and blood pressure changes.

Subject.	Pulse rise. Immediately after exertion. A	B	28′ after exertion. A	B	Systolic pressure rise (— = fall). 1′ after exertion. A	B	29′ after exertion. A	B	Diastolic pressure change (+rise, —fall). 1′ after exertion. A	B	29′ after exertion. A	B
1.	78	74	24	22	10	8	+4	— 9	—16	—1	—18	—1
6.	37	38	9	9	45	50	—9	—12	+ 5	—1	— 7	—9
2.	74	76	25	28	38	28	—7	— 4	+ 8	—1	— 5	could not measure, tailed off
Av.	63	63	19	20	31	29	—4	— 8	— 1	—1	—13	— 5

A=high resistance container. B=low resistance container.

DISCUSSION.

It is evident that unless the exertion be severe, there is little to choose between the containers of the standard resistance and those 0·55 inch water higher. Doubtless this is partly to be explained by the fact that an oxygen debt can be developed. It is well known that a 100 yards race (not in battle kit) can be run almost without taking a breath. The fact that a significant difference was obtained between times for running a 305 yards' course, and that it was not obtained for short bursts of 4 minutes' work on a bicycle ergometer (by different subjects) seems at first difficult of interpretation. The difference may be due to the fact that on a bicycle respiration is greatly aided since the thorax is fixed by the arms clasping the handlebars, and so the muscles of respiration are able to increase their purchasing power. It is known that cyclists can develop a higher horsepower on the bicycle than Olympic crews can when rowing. In running the the arms were holding out a bayonet which at the distance caused some fatigue of certain accessory muscles of respiration, and they were not available for increased respiratory effort. The steep rise in the halfway mark of the course very considerably slowed up each competitor, and further, each individual performed three races one after the other.

The significance of pulse changes would appear now to require reconsideration. More work was done in 20 minutes on the ergometer with the low resistance container and this also was done at the expense of less respiratory effort. However, the pulse rose higher immediately after exertion with the low resistance container (increasing 77 beats compared to 70). With the high resistance container there is an increased negative intrathoracic pressure of approximately 20 p.c. This enables better filling of the heart in diastole and so allows a greater output of the heart per beat which would allow a slower pulse for the same output. The output per minute should give a fair idea of the work done. For the one individual, if the arterio-venous oxygen difference is the same and the efficiency unchanged, the cardiac output should be directly related to the work done. Therefore, assessing the value of containers on pulse changes is a two-edged sword. The effort will increase the pulse, but the increased negative intrathoracic pressure may decrease the pulse rise. The systolic blood pressure changes were similar, rising 11 and 12 mm. Hg. after the exertion on the average with the high and low container respectively. Significantly there was a notable fall half an hour after the exertion (14 mm. Hg.). This was equal in both. However, the blood pressure changes were individually quite variable (Table 6). Further, the changes for the same individual using the one container were also very variable. It would appear that comparative information is not so likely to be obtained from blood pressure changes.

SUMMARY.

For short bursts of work on a bicycle ergometer (4 minutes) there is no significant difference in the work done with low resistance (2·7–2·8 in.) containers and high resistance (3·2–3·3 in.) containers.

For three individual runs over a course of 305 yards, including a steep rise and wearing battle dress with equipment and bayonet fixed, the difference in time was 2·9 p.c.; and for work done on a bicycle ergometer (20 minutes) in battle dress with a 10 lb. pack on the back, the difference in work done with the two different containers was 3·4 p.c. These differences are shown to be highly significant.

When running in relay was performed the times for each container were closer. The reason for this is discussed.

The value of changes in the pulse rate and blood pressure as indices for comparison has been discussed.

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